

THE PREPARATION AND PROPERTIES  
OF ISOLATED CHICKEN HEPATOCYTES.

BY

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PREFACE

The studies reported in this dissertation were carried out in the Veterinary Unit of the Department of Biochemistry in the University of Edinburgh from October 1973 until September 1976. No part of this thesis has been submitted to any other University.

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Conventions and Abbreviations

The conventions of the Biochemical Journal with regard to units and abbreviations have been used throughout. Additional abbreviations are indicated in the text.



ABSTRACT

Chicken hepatic parenchymal cell suspensions, isolated by an optimised collagenase digestion, were used for a study of hepatic glucose metabolism and its control in the chicken. Characterisation of this in vitro preparation showed the parenchymal cells immediately after isolation to be similar to those of whole liver, both morphologically and metabolically. This similarity suggested that metabolic studies with isolated hepatocytes might confidently be extrapolated to the situation in the intact animal. However the preparation quality was dependent on collagenase contaminants and all preparations exhibited decreased viability throughout subsequent incubations.

Glycogen metabolism in isolated hepatocyte suspensions favoured glycogenolysis and under no conditions was net glycogen synthesis observed. Gluconeogenesis from added precursors was difficult to discern with fed chicken hepatocytes due to the high basal glucose production but was readily demonstrated at a constant rate over a two hour incubation with starved chicken hepatocytes.

The gluconeogenic effectiveness of precursors was generally similar in isolated hepatocytes and in chickens in vivo. The greater effectiveness of lactate compared with pyruvate, observed with both systems (unlike the rat), is probably a consequence of impaired hydrogen ion transfer during pyruvate gluconeogenesis due to the mitochondrial location of phosphoenolpyruvate carboxykinase in the chicken. Synergistic interactions between substrates were shown to occur and be important for interpretation of results from isolated hepatocytes for extrapolation to the situation in vivo. Glycerol was

dramatically less effective in vitro than in vivo due to inhibition of glycerokinase activity by the low ATP/ADP ratio of isolated hepatocytes.

Physiological concentrations of glucagon stimulated glycogenolysis and gluconeogenesis from precursors entering the glycolytic pathway above and below the triose phosphate dehydrogenase step. Although it was possible to assign a glucagon control point between triose phosphate and glucose in chicken liver, that between pyruvate and phosphoenol pyruvate (postulated for the rat) was not observed.

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## CHAPTER 1

### GENERAL INTRODUCTION

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### GENERAL INTRODUCTION

#### LIVER FUNCTION

The liver has the most diverse metabolic function of all organs. Although many of its reactions are found in other tissues, certain enzymes are specifically synthesised in liver. The presence of these enzymes gives the liver a unique metabolic profile. The liver contains the enzymes involved in the detoxification of foreign compounds and for the removal of excess nitrogenous compounds from the plasma. The synthesis of urea results in the retention of the carbon skeletons of nitrogenous compounds and their further metabolism by the liver. Apart from their oxidation to carbon dioxide these compounds can also be converted into glucose-6-phosphate. The liver contains enzymes for glycogen synthesis and high contents of glycogen are present when animals have been fed. More important than the ability to store glucose units as glycogen is the presence of glucose-6-phosphatase in the liver. No other tissue, except for kidney, contains glucose-6-phosphatase. Plasma membranes are impermeable to glucose-6-phosphate, and it must be converted to glucose before equilibrating with plasma glucose. Thus only tissues containing glucose-6-phosphatase can release glucose into the plasma, Due to the low glycogen content and smaller size of kidney, liver is quantitatively more important in maintaining plasma glucose.

Although gluconeogenesis occurs through the same intermediates as glycolysis, certain points of the pathway require different enzymes for the forward and backward directions. At these points the net reaction will depend on the relative activities of



enzymes for the forward and back reactions. This has led to the use of the term (and the concept) of "substrate cycling". Such cycles occur at the level of gluco/hexokinase and glucose-6-phosphatase, phosphofructokinase and fructose-1,6-diphosphatase and pyruvate kinase/pyruvate carboxylase and phosphoenolpyruvate carboxykinase. The enzymes required for reversal of glycolysis (glucose-6-phosphatase, fructose-1,6-diphosphatase and phosphoenolpyruvate carboxykinase) are all found in liver. The liver can thus produce glucose either from glycogen or from the net synthesis from any intermediate that can be converted into a glycolytic intermediate.

Certain tissues, especially the brain, normally oxidise only glucose. Dramatic decreases of plasma glucose concentrations would impair brain and nervous tissue function. When glucose supply from the digestive tract ceases during starvation, the plasma glucose concentration is nevertheless maintained. The pancreatic hormones, insulin and glucagon, play a major role in controlling glucose metabolism. Insulin injection to rats in vivo decreases plasma glucose concentrations. This is primarily due to increased glucose uptake by liver, skeletal muscle and adipose tissue either by stimulation of transport processes or of the activity of glucose phosphorylating and glycogen synthesising enzymes. On the other hand, glucagon increases plasma glucose concentration. This is mainly due to effects on the liver, stimulating both glycogenolysis and gluconeogenesis. The effects of insulin and glucagon are antagonistic on hepatic glucose metabolism.

## CHICKEN LIVER

### Metabolism of glucose

Quantitative differences exist between mammalian and chicken livers. The avian liver is the major site of lipid synthesis, whereas in most mammals this function is mainly located in adipose tissue. (Leveille, et al, 1968; O'Hea & Leveille, 1968). The high lipogenic capacity of chicken liver may alter the role or importance of glucose metabolism. Due to the interrelationship of glucose and lipid metabolism a large proportion of glucose metabolism may be to supply fatty acid precursors.

The rates of metabolic pathways are often controlled by the first reaction of a sequence (Krebs, 1957). The metabolism of glucose requires phosphorylation initially. Chicken liver does not contain glucokinase and glucose uptake is dependant on a substrate-cycling system between low  $K_M$  hexokinases and glucose-6-phosphatase (Ureta, et al, 1973; O'Neill & Langslow, 1976). The high plasma glucose concentration (12-15mM) in chickens plus the different enzyme profile between rat and chicken liver may result in different rates of glucose uptake. The rate of metabolism of other sugars has been investigated by their amount of stimulation of oxygen consumption (Heald, 1963). Chicken liver slices metabolise fructose more rapidly than glucose and, unlike rat liver, do not metabolise sorbitol.

The metabolic characteristics of chicken liver have been investigated by analysing the activity and intracellular location of enzymes. Homogenates of chicken embryos contain largely the same enzymes and intermediates of glucose metabolism as the rat

(Stumpf, 1947; Wenger & Kitos, 1967). Chicken liver slices metabolised  $^{14}\text{C}$ -glucose to  $^{14}\text{C}$ -carbon dioxide (Annison, et al, 1966) and synthesised glucose from several precursors (Yarnell, et al, 1966; Bannister, et al, 1975). Qualitatively the available pathways of glucose metabolism and its metabolic fates appear similar in chicken and mammalian liver.

Although the pathways of glycogen metabolism are similar (Hazelwood, 1972), chicken liver contains less glycogen than rat liver (Langslow & Hales, 1971). The activities of the pentose phosphate pathway enzymes decrease after hatching and are very low in 3-4 week old chickens (Duncan & Common, 1967; Duncan, 1968; Goodridge, 1968; O'Hea & Leveille, 1968). In rat adipose tissue, the rates of fatty acid synthesis and the pentose phosphate pathway are correlated through NADPH utilisation and supply (Flatt & Ball, 1966; Katz, et al, 1966). In order to synthesise lipid at the observed rates, chicken liver would require large amounts of NADPH. As this can not be supplied by the dehydrogenases of the pentose phosphate pathway, other enzymes have been implicated, and malic enzyme has been shown to supply a large proportion of the NADPH in chicken liver (Goodridge, 1969).

The intracellular location of phosphoenolpyruvate carboxykinase (E.C. 4.1.1.32) is species dependent (Utter, 1959). In the chicken liver 90% is mitochondrial, in the rat 90% is cytoplasmic and the human liver contains 50% in each compartment. Although the pathways of glucose metabolism are similar in different species, the relative rates and the control of its metabolism may be greatly altered by differences in the activity of enzymes and their intracellular location.

## Control of Glucose Metabolism

Most data on the control of glucose metabolism has been obtained from studies in vivo. The difficulties of extrapolating results from experiments in vivo to effects on the liver are discussed in a later section. Thus although in specific instances the site of control is apparent, conclusions as to control at the hepatic level must be made with extreme care.

Livers isolated from starved chickens utilise glucose at 10% of the rate of fed chicken livers (Annison, et al, 1966; Bickerstaffe, et al, 1970). During starvation plasma free fatty acid concentrations doubled (Lepkovsky, et al, 1967; Langslow, et al, 1970). In mammals glucose uptake by peripheral tissues is reduced during starvation and plasma free fatty acid concentrations increase. Fatty acids are the major metabolites oxidised by most tissues during starvation.

During starvation chickens lose weight due to protein degradation and the increased plasma uric acid concentration suggests that amino acid catabolism has increased, probably via provision of carbon skeletons for gluconeogenesis (Hazelwood & Lorenz, 1959; Lepkovsky, et al, 1967; Langslow, et al, 1970). The injection of gluconeogenic precursors (glycerol, lactate, malate, and to a lesser extent, pyruvate and alanine) into starved chickens elevates plasma glucose concentration (Sarkar, 1971; Davison & Langslow, 1975).

Pancreatic hormones have been injected into chickens to simulate the changes in concentration known to occur in mammals during nutritional changes. Although insulin injection decreases the plasma glucose concentration, the effects are less severe compared to the effects of a similar dose in mammals (Hazelwood & Lorenz, 1959;

Hazelwood, et al, 1968; Langslow, et al, 1970). Insulin antibody injection causes marked hyperglycaemia in chickens (Bondareva, 1970) and stresses the necessity of circulating insulin in chickens.

Glucagon injection increased the plasma glucose concentration and decreased liver glycogen (Heald, et al, 1965; Grande, 1968; Langslow, et al, 1970). The effects of glucagon on plasma glucose are extremely potent (Langslow & Hales, 1971) and this may correlate with the greater sensitivity of isolated chicken fat cells to glucagon compared to the effects on rat fat cells (Langslow & Hales, 1971). Thus, compared to the typical mammalian responses, in terms of effects on plasma glucose concentrations, chickens are more sensitive to glucagon and less sensitive to insulin.

Total pancreatectomy of dogs and rats resulted in greatly increased plasma glucose concentrations but it has been observed many times that pancreatectomy <sup>in chickens</sup> does not result in any dramatic alterations of plasma glucose concentrations (Minkowski, 1893; Hazelwood, 1965; Langslow & Freeman, 1972; Colca & Hazelwood, 1976). Interpretation of the effects of this ablation is difficult in chickens. The pancreas contains not only cells secreting insulin but also others secreting glucagon, somatostatin and avian polypeptide (Kimmel, et al, 1968) and total removal of the pancreas is difficult (Miahle, 1969).

The anomalies surrounding the roles of insulin and glucagon in controlling plasma glucose concentrations suggest that although their actions may be qualitatively similar in mammals and chickens, the effects are quantitatively different. The liver is a major site of pancreatic hormone action and the altered sensitivities may be due to different hepatic level effects of insulin and glucagon.

## TECHNIQUES FOR STUDYING LIVER METABOLISM

### Studies in vivo

The effects of metabolite and hormone injection into animals can be difficult to interpret. The site of action may be uncertain and specific hepatic effects may not occur. This can be partially overcome by liver removal from groups of experimental animals at time intervals after injection and the measurement of cellular metabolites and enzyme activities. However the interaction of injected materials with plasma constituents will greatly influence their actions.

### Studies in vitro

In these systems, specific effects on liver can be studied and the conditions rigorously defined. However, the stresses during removal of the liver from the animal may disturb the organs physiological balance and limit the usefulness of the preparation. Thus the physiological characteristics of the isolated liver preparation and the physiological roles of the whole liver should always be compared whenever possible to assess the quality of the isolated preparation.

- a) **HOMOGENATES:** Homogenates and subcellular fractions of liver with added cofactors are commonly used to measure enzyme activities. However as this system has lost its physical integrity it is not suitable for studying biosynthetic processes and their control.
- b) **ISOLATED PERFUSED LIVER:** These preparations are comparable to whole liver in terms of ultrastructure, hormonal sensitivity, contents of adenylates,  $K^+$  and glycogen and lack of enzyme leakage (Mortimore, 1961; Hems, et al, 1966; Ross, et al, 1967; Exton & Park, 1969). They carry out many of the biosynthetic reactions due to the retention of multienzyme systems, cellular compartmentation and physiological integrity. Isolated perfused livers are stable for several hours and monitoring of oxygen

consumption indicates the onset of degeneration. The system is limited by the difficulty in supplying sufficient oxygen to the tissue during perfusion and by the few experimental conditions that can be studied with each liver. Interpretation of results is complicated by the presence of parenchymal and nonparenchymal cells in the organ.

- c) LIVER SLICES: Although several variables can be studied simultaneously using liver slices (Krebs, et al, 1966), this rapid and simple technique has few advantages and many disadvantages. Slices are inevitably of different thicknesses and the diffusion of metabolites in and out of the tissue is limited. Slicing damages the outer cells of each slice, causes the loss of adenylates and alters the tissue redox state (Krebs, et al, 1974). Stimulation of gluconeogenesis by glucagon is absent in rat liver slices although phosphorylase activity is increased (Miller, et al, 1974). The characteristics of liver slices suggests that they are never the method of choice for studying liver metabolism or its control.
- d) ISOLATED HEPATOCYTE SUSPENSIONS: The earliest attempts to isolate liver cell suspensions relied on mechanical dispersion of the cells with loose homogenisers (Potter & Elvehjem, 1936). To facilitate the isolation and lessen the mechanical stresses, Anderson (1953) perfused the liver with calcium-chelating agents to promote the breakdown of tight junctions. However the cells remained enmeshed in intracellular connective tissue and still required mechanical force for dispersion. The damage caused by these stresses resulted in suspensions of metabolically inert cells (Berry, 1962).

Nonspecific proteases (such as trypsin) were used initially to break down the intercellular matrix. Although this method was



used by some people (Bissel & Tilles, 1971; Montavalo, et al, 1972) it was not favoured due to the protease damage to membranes and to hormone receptors (Aubin & Bucher, 1952; Gunther & Goecke, 1966).

Rodbell (1964) used collagenase to digest connective tissue in order to isolate rat adipose tissue cells. This was used as the basis for the isolation of rat hepatocytes (Howard, et al, 1967). Their method involved the use of collagenase and hyaluronidase digestion of liver slices and has since been refined (Howard & Pesch, 1968; Howard, et al, 1973). Rat hepatocytes were also isolated after the recirculating perfusion of livers with a solution of collagenase and hyaluronidase (Berry & Friend, 1969). Although the latter method, and its subsequent modifications, results in yields greater than from slice digestion, it requires all the equipment associated with liver perfusion (Capuzzi, et al, 1971; Seglen, 1972, 1973a, 1973b; Cornell, et al, 1974). These original methods have been modified to isolate hepatocytes from several other species.

Suspensions of isolated hepatocytes have several advantages over other liver preparations in vitro. Suspensions consist almost entirely of parenchymal cells and this overcomes the heterogeneity associated with perfused liver and liver slices. Several experimental conditions, each with internal controls, can be studied from the same liver. Diffusion problems are overcome by the nature of the suspension and oxygen will be readily available to all cells.

However, membranes may be damaged by the digestive enzymes resulting not only in gross damage (loss of cell content) but also more subtle damage (loss of hormone sensitivity). The increased



surface area after isolation may cause greater permeability to hepatocyte metabolites. Preparations must be oxygenated during isolation to prevent the loss of labile metabolites resulting from anoxia. Cell-cell contacts may stabilise hepatocytes in situ and their loss after hepatocyte isolation may stimulate degenerative changes in cell structure. The limitations imposed by these factors on the usefulness of isolated hepatocytes requires further investigation.

#### AIM AND EXPERIMENTAL APPROACH

Although the metabolic pathways of chicken liver are similar to those of mammalian liver, there has been no comprehensive study of glucose metabolism or of the action of pancreatic hormones at the hepatic level. Isolated hepatocytes appeared to be ideally suited for studying metabolism and its control. At the start of my project, Goodridge (1973) had used collagenase to isolate hepatocytes from day-old chicks. These hepatocytes had an extremely high lipid content. In this paper there was a brief mention of the author having isolated hepatocytes from four-week old chickens (an age when embryonic metabolic profiles have been lost, Hazelwood, 1972). Little information was included on the quality of the preparation. Many authors stress the importance of high yields, but it is the quality rather than the quantity that determines the usefulness of isolated cell preparations. Too frequently, reports on the metabolism of isolated hepatocytes give little or no indication as to the quality of the preparations.

The isolation of hepatocytes by slice digestion appeared relatively simple and required no specialised equipment. This method

was therefore modified for the isolation of chicken hepatocytes. Biochemical and morphological similarities between isolated hepatocytes and whole chicken liver were used to assess the quality of the preparation. From the comparison of several structural and metabolic characteristics, a method was developed for routine estimation of the physiological integrity of preparations.

Physiologically competent hepatocytes were used to study the metabolism of glucose and glycogen in chicken liver and to study gluconeogenesis from a wide variety of exogenous substrates. The metabolic capacity of chicken liver and control points of gluconeogenesis were compared to those of other species. Finally, the effects and mechanism of action of glucagon on glycogenolysis and gluconeogenesis were investigated.

## CHAPTER 2

### PREPARATION AND MORPHOLOGY OF ISOLATED CHICKEN HEPATOCYTES

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### INTRODUCTION

This chapter describes the method evolved for preparing isolated chicken liver cells and for assessing their morphological integrity. The problems associated with variations in the properties of collagenase supplied by Boehringer and with the age of chickens are discussed.

### PREPARATION OF ISOLATED HEPATOCYTES

The procedure was an *modification* of Howard and Pesch's (1968) method, and incorporates the modification described by Howard, *et al*, (1973). The efficiency of hepatocyte isolation is decreased by the presence of  $\text{Ca}^{2+}$  (Howard, *et al*, 1973), and thus hepatocytes were prepared in  $\text{Ca}^{2+}$ -free Krebs-Ringer bicarbonate solution ( $\text{Ca}^{2+}$ -free KRB). The subsequent incubations were carried out in normal Krebs-Ringer bicarbonate (KRB) (Krebs & Hensleit, 1932). Before use the Krebs-Ringer bicarbonate solutions were thoroughly oxygenated by gassing with 95%  $\text{O}_2$ : 5%  $\text{CO}_2$  until the pH equilibrated at 7.4. Bovine serum albumin (BSA) was added to all buffers (2% w/v). This addition stabilises hepatocytes (Krebs, *et al*, 1974). All glassware was siliconised by immersion in Repelcote followed by thorough washing in distilled water and drying. The possible destruction of hepatocytes by adherence to glass surfaces was prevented by this treatment.

Sources of animals and chemicals are detailed in Appendix 2.

### Perfusion and slicing of liver

The perfusion apparatus consisted of a three-way tap connected by polypropylene tubing to two 10 ml plastic syringe bodies and a number 19 gauge needle. This permitted liver perfusion under gravity by one of two solutions. Assistance was required for the switching from one reservoir to the other.

Chickens were killed by cervical dislocation. Following incisions on either side of the rib cage from the carina to the backbone, the rib cage was pulled upwards and the liver exposed. The bile ducts were teased gently away from the hepatic portal vein and cut, and the portal vein was fully exposed by the removal of connective tissue and fat from the surrounding area. The needle was inserted into the portal vein, the inferior vena cava cut immediately and the liver perfused in situ with 10 ml of ice-cold  $\text{Ca}^{2+}$ -free KRB. The liver was not handled prior to perfusion as contact resulted in clotting. Due to the rapid clotting of chicken blood these parts did not subsequently perfuse. If the needle is successfully located in the vein the blood is washed out and a rapid, even blanching and cooling of the liver results during the perfusion. If these criteria were fulfilled, the perfusion was continued with 10 ml of ice-cold  $\text{Ca}^{2+}$ -free KRB containing 0.25 mg collagenase and 1.0 mg hyaluronidase per ml. When the perfusate was finished, the liver was excised, weighed and sliced (0.5<sub>mm</sub> thick slices) with a Stadie-Riggs hand tissue slicer (Arthur H. Thomson, Co., Philadelphia, U.S.A.).

### Isolations and purification of hepatocytes

The liver slices, and a further 20 ml of  $\text{Ca}^{2+}$ -free KRB containing the digestive enzymes in a 100 ml siliconised flask,

were shaken (100 cycles/min) with constant gassing in a metabolic water bath at 40°C (close to the body temperature of the chicken). After 60 minutes, 30 ml of ice-cold  $\text{Ca}^{2+}$ -free KRB was added and, with a gentle swirling motion, the suspension was filtered first through a coarse filter and then through a fine nylon mesh (150  $\mu$  pore size). The material retained in the filters was discarded and the filtrate was centrifuged in polypropylene tubes at 335 g for 5 minutes, then at 200 g, 110 g and 60 g for 2 minutes each. At each stage the supernatant was discarded and the pellet gently resuspended in 20 ml ice-cold  $\text{Ca}^{2+}$ -free KRB with a wide tipped pasteur pipette. The final pellet was resuspended in the required volume of KRB and either analysed immediately or incubated for subsequent analysis. Hepatocyte suspensions (0.5 ml aliquots in quadruplicate) were added to 1.0 ml KRB in stoppered polypropylene pots, gassed for 30 seconds with 95%  $\text{O}_2$ : 5%  $\text{CO}_2$  and incubated at 40°C with gentle shaking (40 cycles/min). For incubations of more than one hour, hepatocytes were regassed for 30 seconds each hour.

#### MORPHOLOGY OF ISOLATED HEPATOCYTES

##### Light microscopic studies

Yields of hepatocytes isolated by enzymic digestions have frequently been determined by counting aliquots of suspension under the light microscope (Howard & Pesch, 1968; Berry & Friend, 1969). Vital dyes such as trypan blue stain nuclei (Hoskins, et al, 1956) and trypan blue is used to indicate gross structural damage to hepatocytes as it will only stain nuclei of hepatocytes with damaged plasma membranes (Howard, et al, 1967; Berry, 1974). Aliquots (0.2 ml) of suspensions were routinely added to 0.8 ml of 1% trypan blue in isotonic KCl and drops on a haemocytometer grid were examined by light microscopy for estimation of both the yield and the extent of damage of the preparations

TABLE 2.1. Effect of varied concentrations of hyaluronidase on yield and percentage of trypan blue stained hepatocytes.

A. <u>Yield</u>		<u>Hepatocyte/g liver (X10<sup>6</sup>)</u>	
		<u>FED CHICKENS</u>	<u>24h STARVED CHICKENS</u>
0.25 mg Collagenase/ml only		29.2 ± 2.9 (41)	23.0 ± 4.2 (13)
_____ plus		-	
_____ 0.25 mg Hyaluronidase/ml			33.8 ± 3.6 (17)
_____ plus			-
_____ 0.5 mg Hyaluronidase/ml		36.8 ± 7.3 (7)	
_____ plus			
_____ 1.0 mg Hyaluronidase/ml		34.0 ± 4.3 (22)	32.2 ± 4.7 (17)
B. <u>Percentage initially stained</u>			
		<u>Percentage Stained</u>	
		<u>FED CHICKENS</u>	<u>24h STARVED CHICKENS</u>
0.25 mg Collagenase/ml only		24.4 ± 2.1 (41)*	33.6 ± 4.6 (13)*
_____ plus		-	
_____ 0.25 mg Hyaluronidase/ml			26.9 ± 2.8 (17)
_____ plus			-
_____ 0.5 mg Hyaluronidase/ml		15.9 ± 6.6 (7)	
_____ plus			
_____ 1.0 mg Hyaluronidase/ml		20.6 ± 2.0 (22)**	27.8 ± 2.7 (17)**

Results are means ± SEM's with the number of experiments in parentheses. No statistically significant differences except for - \* P < 0.025; \*\* P < 0.05.

TABLE 2.2. Effects of centrifugation on cellular composition of parenchymal cell preparations.

Cell counts were performed on aliquots either from the final cell suspension or from supernatants after each centrifugation. Results expressed are from one experiment and are total cells ( $\times 10^6$ ) for each fraction.

<u>STAGE</u>	<u>Unstained</u> <u>parenchymal</u> <u>cells</u>	<u>Stained</u> <u>parenchymal</u> <u>cells</u>	<u>Red</u> <u>blood</u> <u>cells</u>	<u>Non-</u> <u>parenchymal</u> <u>cells</u>
335g supernatant	ZERO	ZERO	0.22	0.11
200g Supernatant	ZERO	0.35	0.06	2.32
110g Supernatant	0.16	0.92	0.32	3.19
60g Supernatant	0.10	1.25	0.30	3.55
Final cell suspension	26.08	8.05	3.68	0.56



(Table 2.1). At low magnification (x100), the suspensions

consisted almost exclusively of parenchymal cells, mostly singly but occasionally in clumps of 3 or 4. The differences in structure between hepatocytes with stained nuclei and unstained hepatocytes was more obvious under higher magnification (x400). Parenchymal cells with stained nuclei had a disorganised appearance. The cytoplasm appeared translucent and empty and there was no obvious plasma membrane. Unstained parenchymal cells retained a dense granular cytoplasm and a spherical shape enclosed within a refractile plasma membrane.

Contamination from other cell types was minimal. Only 0 to 2 percent of the liver cells present were nonparenchymal. Küpffer cells were densely stained throughout the cytoplasm and nucleus with trypan blue. Endothelial cells are about one third of the diameter of hepatocytes and appeared as granular spheres. The contamination by oval nucleated blood cells depended greatly on the perfusion and, to a lesser extent, on the washing during isolation. Normally, red blood cells accounted for only 3-8 percent of the total number of cells present.

The supernatants discarded during the washing stages of isolation were examined under the light microscope (Table 2.2). Although a few parenchymal cells were lost in these supernatants, the vast majority were retained in the pellet fraction. Washing mostly removed debris, nonparenchymal cells and red blood cells.

The diameters of chicken parenchymal cells were determined by examination of 5 individual hepatocyte preparations under oil immersion (x1000). Using an eyepiece graticule, 200 parenchymal cells from each preparation were examined and diameters were in the range 10.5 to 12.0  $\mu$  (mean 11.1  $\mu$ ).

### Electron microscopy

Immediately after isolation, 5 ml aliquots of suspension were centrifuged (30 s at 2750 g) and the pellets were fixed in 1% glutaraldehyde for 30 minutes. The cells were then centrifuged, washed with isotonic phosphate solution (pH 7.4) and recentrifuged. The pellet was resuspended in 2-3 drops of agar solution (2% w/v). After this solidified on a glass slide, cubes (1 mm) were cut with a razor blade and placed in osmium tetroxide (1% w/v) for 30 minutes. The cubes were separated from the solution by centrifugation and were placed in ethanol. The subsequent washing, dehydration, embedding, sectioning and lead citrate staining were carried out by Mr. Neil Smith, Department of Anatomy, Royal (Dick) School of Veterinary Studies, Edinburgh. Thin slivers of liver (1 mm thick) were removed from the liver immediately after perfusion and sections prepared for electron microscopic study using a method similar to that for cells.

Under the electron microscope most cells had an appearance<sup>similar</sup> to parenchymal cells in the whole liver (Figs. 2.1 and 2.2). They had intact plasma membranes, organised nuclei and nucleoli, granular cytoplasm and well preserved mitochondria (Figs. 2.3, 2.4 and 2.5). However, a percentage of the parenchymal cells exhibited signs of damage (Howard, et al, 1967 and 1973; Berry & Friend, 1969; Berry, 1974) with disrupted nuclei and plasma membranes and swollen mitochondria (Figs. 2.6, 2.7 and 2.8). Whether these damaged hepatocytes corresponded to trypan blue stained hepatocytes is not certain. It is not possible to estimate how much damage occurred to hepatocytes during the processes involved in preparing sections and interpretation is extremely difficult.

FIGURE 2.1

Electronmicrograph of liver sections  
from fed chickens.

Magnification - x 12,500

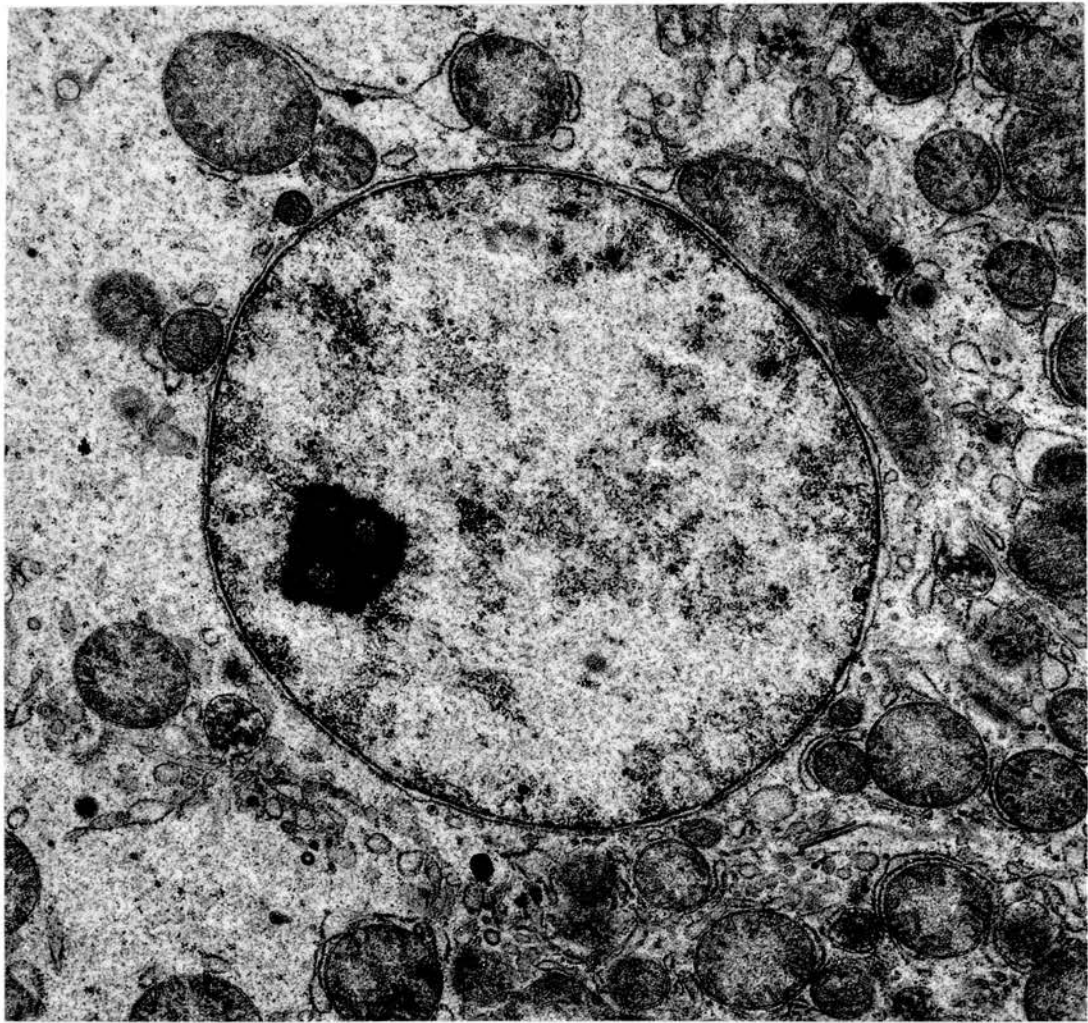


FIGURE 2.2

Electronmicrograph of liver sections  
from 24 hour starved chickens

Magnification - x 12,500

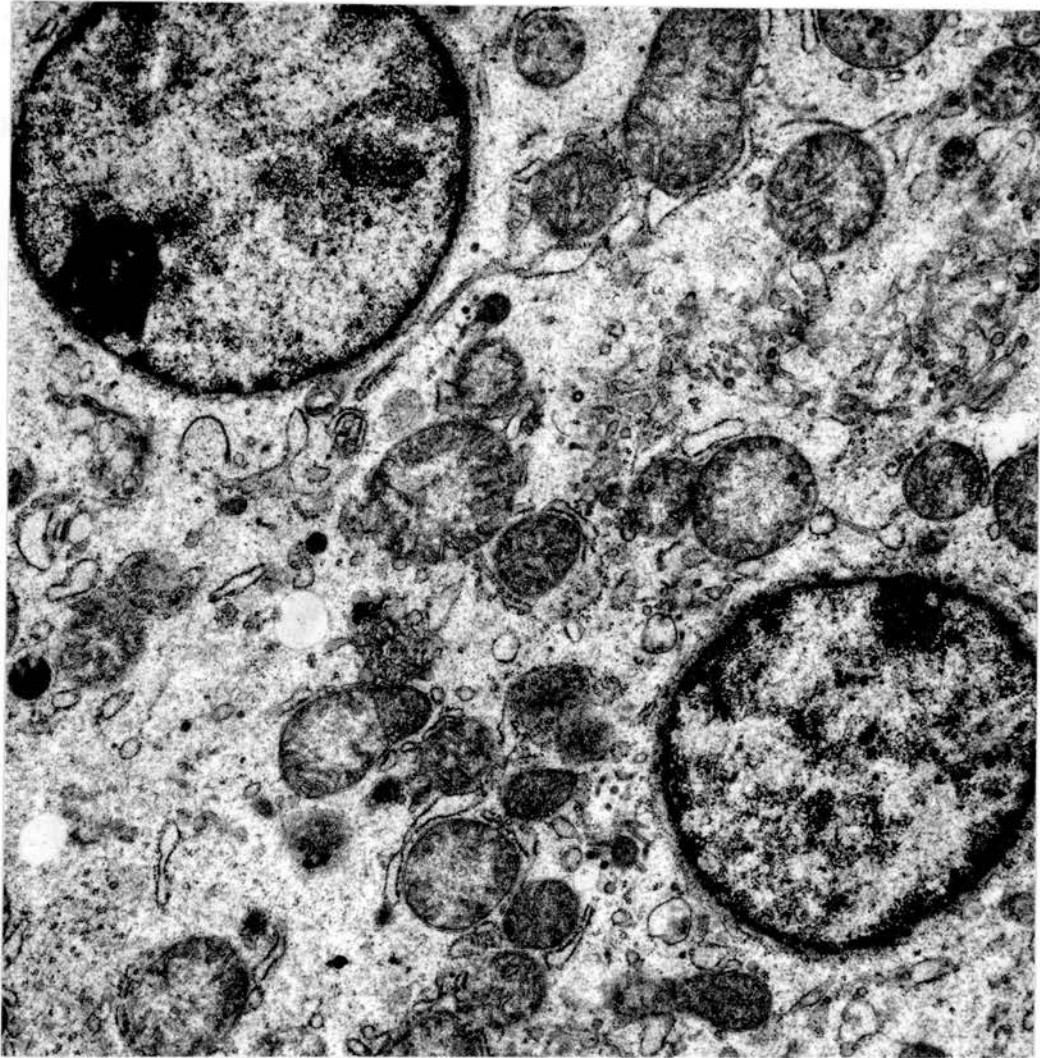


FIGURE 2.3

Electronmicrograph of hepatocyte  
isolated from 24 hour starved chicken

Magnification - x 12,500





FIGURE 2.4

Electronmicrograph of hepatocyte  
isolated from 24 hour starved chicken

Magnification - x 12,500

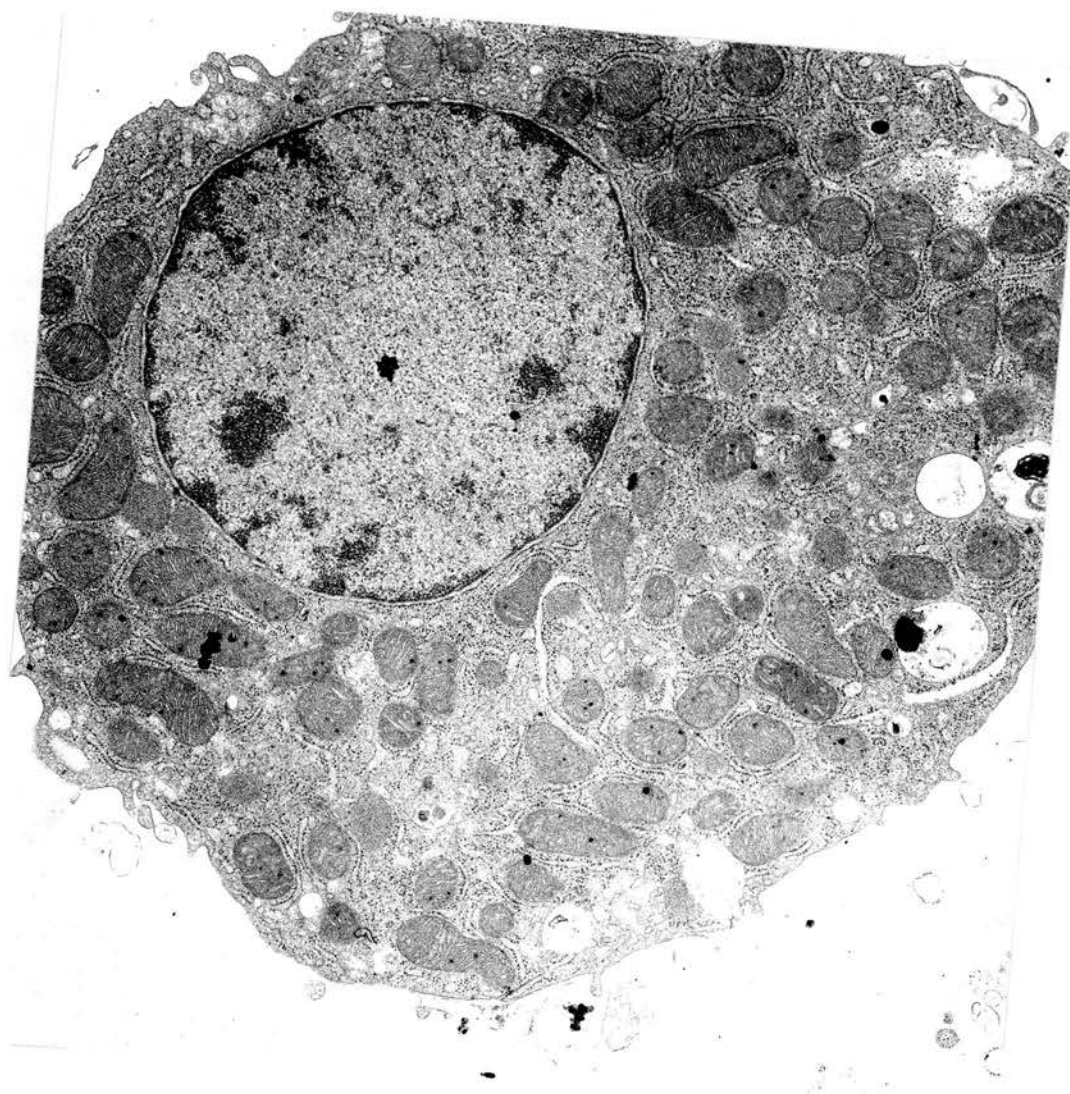


FIGURE 2.5

Electronmicrograph of hepatocytes  
isolated from 24 hour starved chickens

Detail shown of point of contact between two  
isolated hepatocytes.

Magnification - x 50,000



FIGURE 2.6

Electronmicrograph of hepatocyte  
isolated from 24 hour starved chicken

Magnification - x 12,500

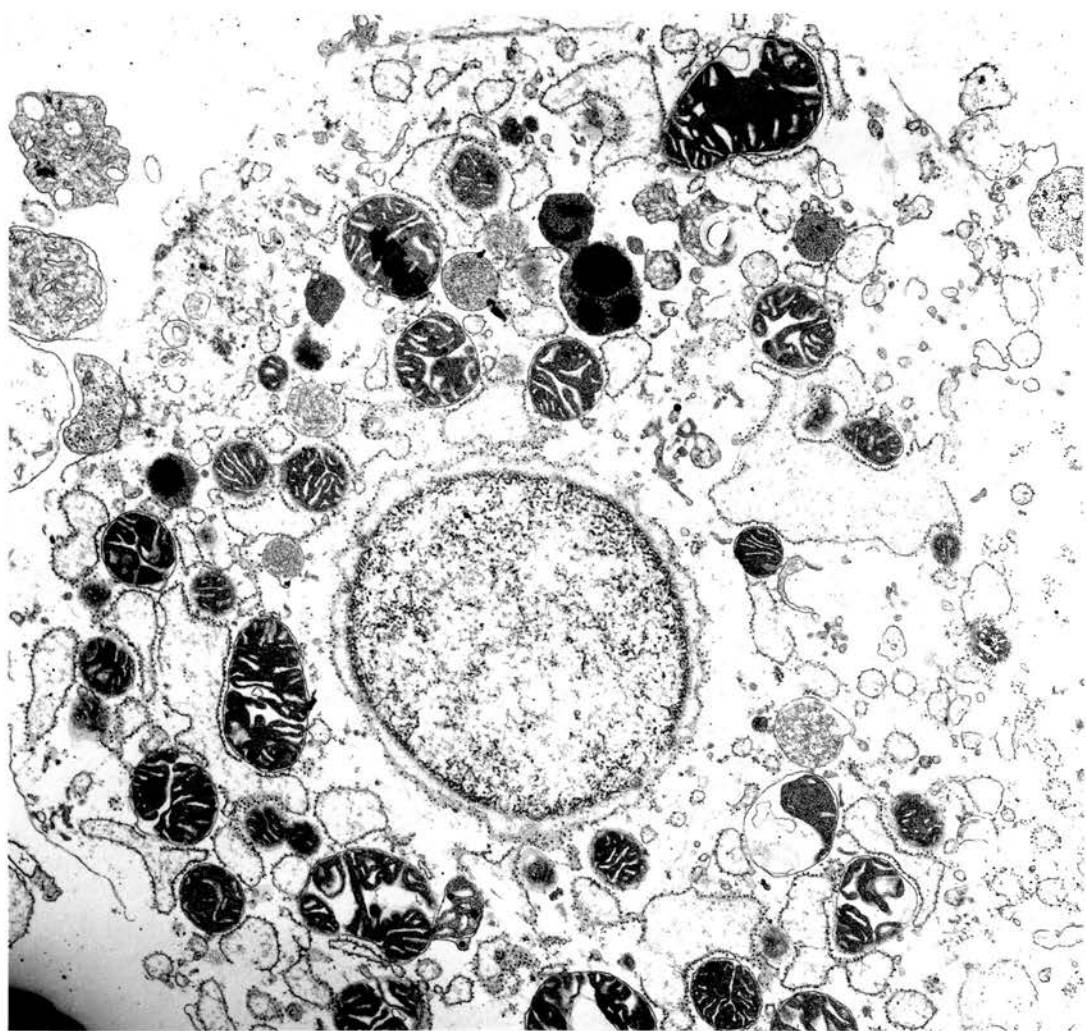


FIGURE 2.7

Electronmicrograph of hepatocyte  
isolated from 24 hour starved chicken

Magnification - x 16,750

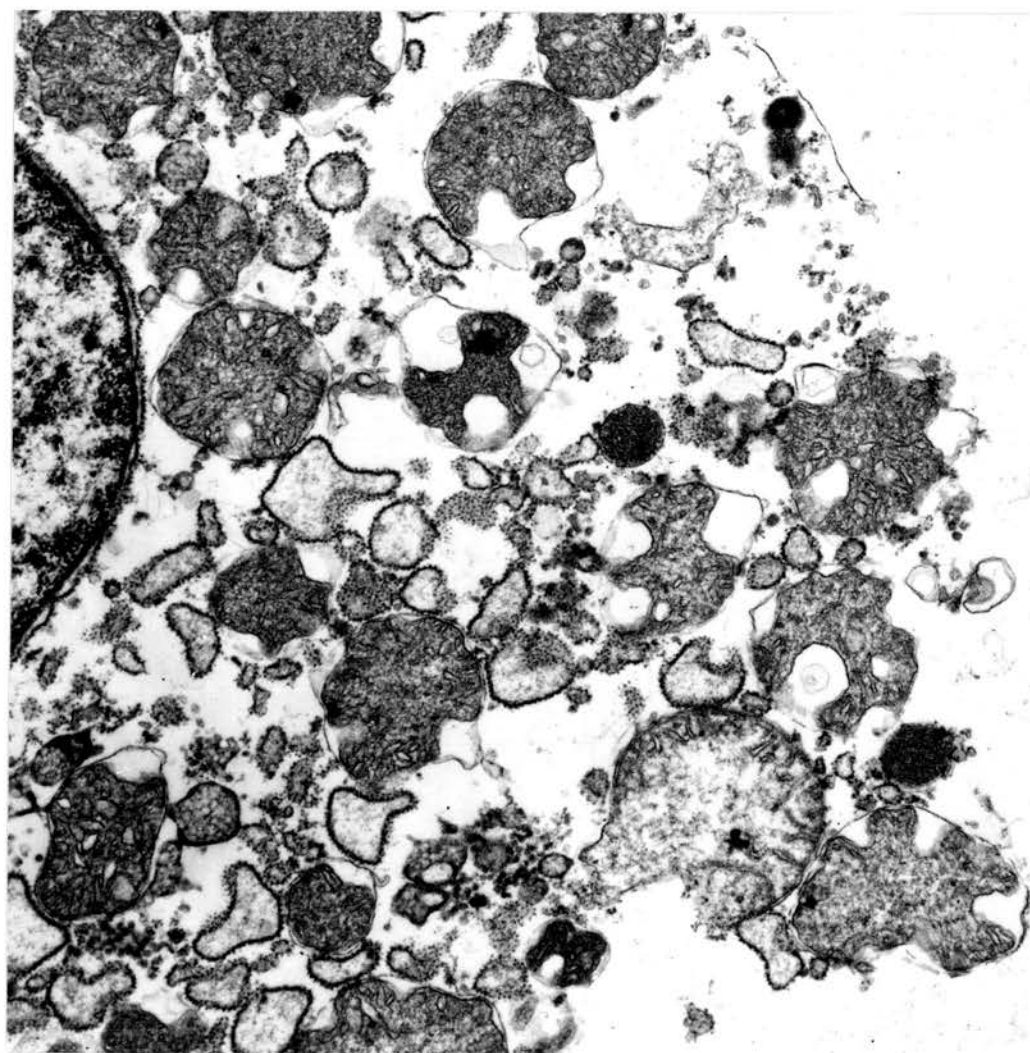
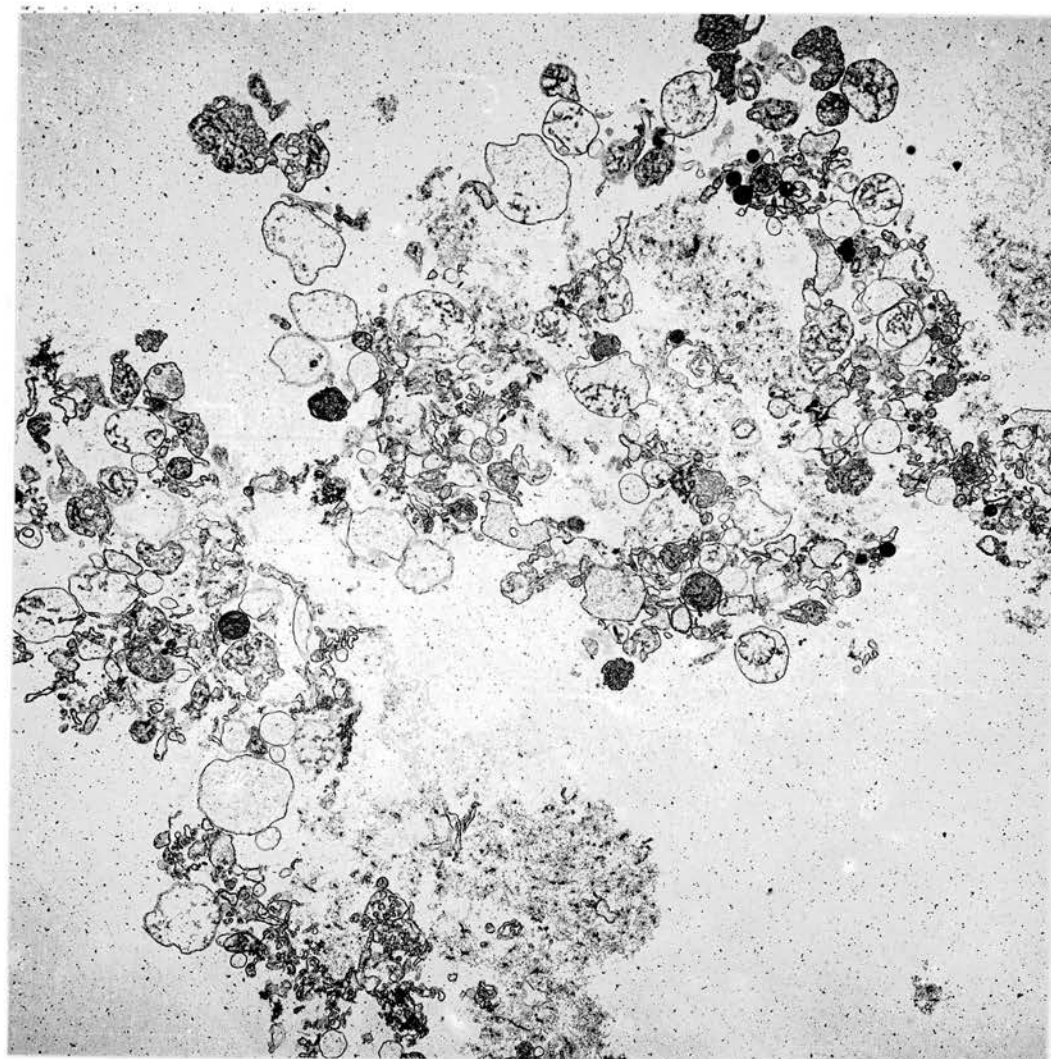




FIGURE 2.8

Electronmicrograph of cellular debris in  
hepatocyte preparation isolated from 24  
hour starved chickens.

Magnification - x 12,500



## OPTIMISATION OF ISOLATION CONDITIONS

The method of Howard and Pesch (1968) was originally used for the isolation of rat hepatocytes. Although their basic method can be used to isolate chicken hepatocytes (Table 2.1), the conditions may not be ideal for chicken hepatocytes. Thus the different stages of <sup>the</sup> isolation procedure were studied in order to optimise them for the preparation of chicken hepatocytes.

The presence of hyaluronidase during the preparation of hepatocytes had no significant effect on the yield of cells or on the percentage of hepatocytes initially stained with trypan blue (Table 2.1). As will be discussed in Chapter 3, hyaluronidase had no effect on a number of metabolic characteristics and therefore was omitted from all subsequent preparations. Similar observations have now been made by several groups isolating rat hepatocytes and most workers isolate hepatocytes with collagenase only (Krebs & Söling, 1976).

Although yields were similar, the initial percentage of trypan blue stained cells was significantly greater for preparations from starved chickens compared to those from fed chickens (Table 2.1). This occurred whether hyaluronidase was present or absent.

Yields could be increased by a longer incubation with collagenase but there was a concomitant increase in the percentage of stained hepatocytes (Table 2.3). Incubation of slices with collagenase for 60 minutes provided an optimal balance between increased yield and a minimum number of damaged hepatocytes, and was used for subsequent preparations involving slicing. Perfusion with  $\text{Ca}^{2+}$ -free KRB weakens

TABLE 2.3. Effect of time of enzyme digestion on hepatocyte yield and percentage initially stained with trypan blue.

One representative set of results are presented for hepatocytes prepared from a fed chicken with 0.25 mg collagenase/ml

<u>Time of incubation</u>		
<u>with collagenase</u>	<u>Yield(X10<sup>6</sup>/g liver)</u>	<u>% Stained</u>
<u>(min)</u>		
30	12.14	8.1
60	33.52	14.8
90	42.30	23.2

the intercellular junctions and hepatocytes can be separated by gentle mechanical treatment, thus lessening the stress applied to hepatocytes during isolation (Berry, 1974). To facilitate this loosening of junctions,  $\text{Ca}^{2+}$ -chelating agents have been perfused through the liver prior to the addition of collagenase (Berry & Friend, 1969).

Pretreatment of livers from fed chickens with EDTA (1 mM) before the addition of 0.25 mg collagenase/ml resulted in yields of  $26.9 \pm 4.1 \times 10^6$  hepatocytes/g liver of which  $21.3 \pm 4.3\%$  were stained (8 experiments) compared to  $29.2 \times 10^6$  and 24.4% without EDTA. As EDTA addition offered no advantages, it was not used routinely for isolation.

#### EFFECTS OF COLLAGENASE ACTIVITY ON PREPARATION OF HEPATOCYTES

Pure collagenase resulted in poor yields of isolated hepatocytes (Berry, 1974). Collagenase used for isolations contains proteolytic contaminants which facilitate liver digestion (Berry, 1974). Batches of collagenase are prepared on a large scale and their relative contamination varies. Hence their <sup>differing</sup>potency for hepatocyte isolation. An alteration in the potency of collagenase obtained from Boehringer in the middle of my project had profound effects on hepatocyte isolation. Due to an increased activity, slices were digested almost completely during a 60 minute incubation. However the yield of hepatocytes prepared from fed chickens with this collagenase decreased to  $19.9 \pm 3.8 \times 10^6$  hepatocytes/g liver (19 experiments) as a result of total enzymic destruction of hepatocytes. Although there was no significant increase in the percentage of trypan blue stained hepatocytes, their hormonal sensitivity was greatly reduced as, presumably, a result of membrane damage. Shorter incubation with lower collagenase concentrations partially overcame these difficulties but the damage to hormone receptors persisted. Thus the digestion of slices (Method A)

was modified to produce hepatocytes after an absolute minimum of contact time with collagenase (Method B).

#### HEPATOCYTE ISOLATION AFTER MINIMUM COLLAGENASE CONTACT

Livers were perfused with 10 ml  $\text{Ca}^{2+}$ -free KRB followed by 50 ml  $\text{Ca}^{2+}$ -free KRB containing 0.1 mg collagenase/ml. The liver was excised, the capsule peeled off and the liver rapidly minced with scissors. The tissue was placed in a siliconised flask with a further 20 ml of the enzyme solution and weighed. With continuous gassing, the mixture was shaken (100 cycles/min) at  $40^{\circ}\text{C}$  for 5 min, and then filtered through a coarse nylon mesh. The suspension was returned to the water bath for a further 2 min to loosen clumps of hepatocytes and filtered through a fine nylon mesh into 30 ml ice-cold  $\text{Ca}^{2+}$ -free KRB. Hepatocytes were then purified by only two centrifugations (335 g for 5 min and 60 g for 2 min) and were finally resuspended in KRB. The entire procedure required 25 min (Method A - 90 min) and the total contact time with collagenase was only 15 min (Method A - 70 min).

As will be discussed in Chapter 6, hepatocytes prepared by Method B were hormonally sensitive. Not only were the problems caused by the very active collagenase overcome but the modifications also resulted in a great improvement in yield compared to Method A (Table 2.4). Using the same batch of collagenase, yields were increased three fold and the initial percentage of stained hepatocytes decreased by two or three fold.

These modifications increased the quality of the isolated hepatocyte suspensions as judged in terms of trypan blue exclusion. Hepatocytes isolated by either Method A or B were almost identical in

TABLE 2.4. Yield and initial percentage of stained cells of suspensions prepared by method B.

Yield as units  $\times 10^6$  hepatocytes/g liver. Results are means  $\pm$  SEM. Number of experiments in brackets.

<u>Age of chicken (weeks)</u>	<u>FED CHICKENS</u>		<u>24h STARVED CHICKENS</u>	
	<u>Yield</u>	<u>Percentage stained</u>	<u>Yield</u>	<u>Percentage stained</u>
3	105.40 $\pm$ 18.56 (12)	10.4 $\pm$ 1.9	118.18 $\pm$ 17.86 (7)	12.8 $\pm$ 3.1
4	73.86 $\pm$ 7.51 (16)	8.5 $\pm$ 1.2	80.67 $\pm$ 6.28 (46)	13.0 $\pm$ 1.5
5	63.18 $\pm$ 4.16 (14)	16.7 $\pm$ 2.6	65.75 $\pm$ 5.05 (31)	15.0 $\pm$ 1.5
6	55.85 $\pm$ 10.11 (8)	8.9 $\pm$ 0.8	56.33 $\pm$ 10.46 (13)	16.1 $\pm$ 2.4

terms of their metabolic characteristics (see Chapter 3). Apart from the increased hormonal sensitivity (see Chapter 6), method B resulted in hepatocytes with adenylate contents similar to whole liver (Tables 3.4 and 3.5), whereas hepatocytes prepared by Method A had much lower total adenylate contents.

## DISCUSSION

Methods A and B each gave similar relative yields of hepatocytes from fed and 24 h starved chickens, and the hepatocytes from 24 h starved chickens always had a greater percentage of stained cells initially (Tables 2.1 and 2.4). The increased fragility of hepatocytes from starved chickens is commented on in more detail in Chapter 3.

Chickens of 3 to 6 weeks of age are of a convenient size for experimentation and have reached an intermediate plateau between changes in metabolic patterns (Hazelwood, 1972). Post-hatching changes have finished and the alterations associated with the onset of sexual maturity have not yet begun. Hepatocyte yields were greatest for 3 to 4 week old chickens and decreased with older chickens (Table 2.4). Thus to obtain large numbers of hepatocytes, chickens of 3 to 4 weeks were used for isolation.

The yields of hepatocytes produced by Method A were similar to those found by Badenoch-Jones and Buttery (1975) for chicken hepatocytes prepared by slice digestion ( $25-50 \times 10^6$  hepatocytes/g liver). Capuzzi, et al (1974) produced  $51-159 \times 10^6$  chicken hepatocytes/g liver using a recirculating perfusion system and Method B produced similar results. With a knowledge of the dry weight of chicken hepatocytes and



a dry to wet weight conversion factor (see Chapter 3), an approximate value for the hepatocyte yield can be calculated. Although the percentage contribution of parenchymal cells to chicken liver weight is unknown, the conversion suggests that 7-15% of the total liver weight is recovered as isolated parenchymal cells. These yields are lower than those routinely found for isolated rat hepatocytes prepared by recirculating perfusions which are in the region of 50% (Berry & Friend, 1969; Krebs, et al, 1974). Using slice digestion, Howard and Pesch (1968) obtained yields of 7% from rat livers. Thus it is possible that damage caused by slicing (Method A) or cutting (Method B) of liver prior to digestion may lower the yield of hepatocytes.

The initial percentage of hepatocytes excluding vital dyes has been reported between 80 and 98 percent for both isolated chicken and rat hepatocytes (Howard & Pesch, 1968; Berry & Friend, 1969; Capuzzi, et al, 1974; Badenoch-Jones and Buttery, 1975). Isolation of hepatocytes from slices (Method A) resulted in relatively high percentages of stained hepatocytes but Method B produced a suspension of a quality equal to or better than any other preparations.

#### SUMMARY

The method of Howard and Pesch (1968) was greatly modified to produce a method for the rapid isolation of chicken hepatocytes. The advantages of simplicity offered by the original technique are retained. Isolated chicken parenchymal cells are morphologically similar to parenchymal cells in the whole liver. Although the yield is not as great as those obtained with recirculating perfusions, the high quality of the preparation suggests that it will be useful for metabolic studies.

The activity of collagenase has been shown to be of utmost importance for obtaining high quality preparations. This indicates that collagenase batches should be put through screening tests prior to being used for any major series of experiments.

In some experiments hepatocytes were prepared and incubated in a modified KRB with high  $K^+$  and low  $Na^+$ . This buffer differed from normal KRB only by the reversal of the concentrations of  $K^+$  and  $Na^+$  in the medium.

## CHAPTER 3

### CHARACTERISTICS OF ISOLATED CHICKEN HEPATOCYTES.

INTRODUCTION

Morphological studies indicate that enzymatically isolated chicken hepatocytes are similar to those in whole liver. Structural intactness need not correlate with metabolic integrity, and it is essential to study the metabolic characteristics of isolated hepatocytes and compare them to those of whole liver. Thus a number of metabolic characteristics were investigated in whole liver and in isolated hepatocytes immediately after isolation. The quality of the preparations were also investigated during incubations of up to 5 hours since changes in their properties might limit their usefulness.

METHODS USED FOR ASSESSING INTEGRITY

Trypan blue exclusion was measured routinely and correlated with some of the other assessments of integrity.

(I) Measurement of enzyme activities

Aliquots (2 ml ) of cell suspensions were centrifuged (30 s at 2750 g ) and both the supernatant and pellet fractions retained for analysis. The cell pellet was lysed with 2 ml of distilled water to release intracellular enzymes and was recentrifuged after standing for 30 minutes on ice. Pieces of whole liver were removed after perfusion and homogenised in distilled water containing 0.5% Triton X-100. Enzyme activities were unaffected by this concentration of Triton

Activities were measured at 25°C and each sample measured in triplicate.

- (a) LACTATE DEHYDROGENASE (E.C. 1.1.1.26) was measured by the method of Bergmeyer, et al (1965).

- (b) GLUTAMATE DEHYDROGENASE (E.C. 1.4.1.3) was measured by the method of Schmidt (1965) incorporating the modifications for chicken liver enzyme suggested by Snoeke (1956).
- (c) ASPARTATE AMINOTRANSFERASE (E.C. 2.6.1.1) was measured by the method of Bergmeyer and Bernt (1965).

Aliquots from the supernatant fraction of hepatocytes were analysed undiluted but the pellet fraction and the whole liver homeogenates required dilution.

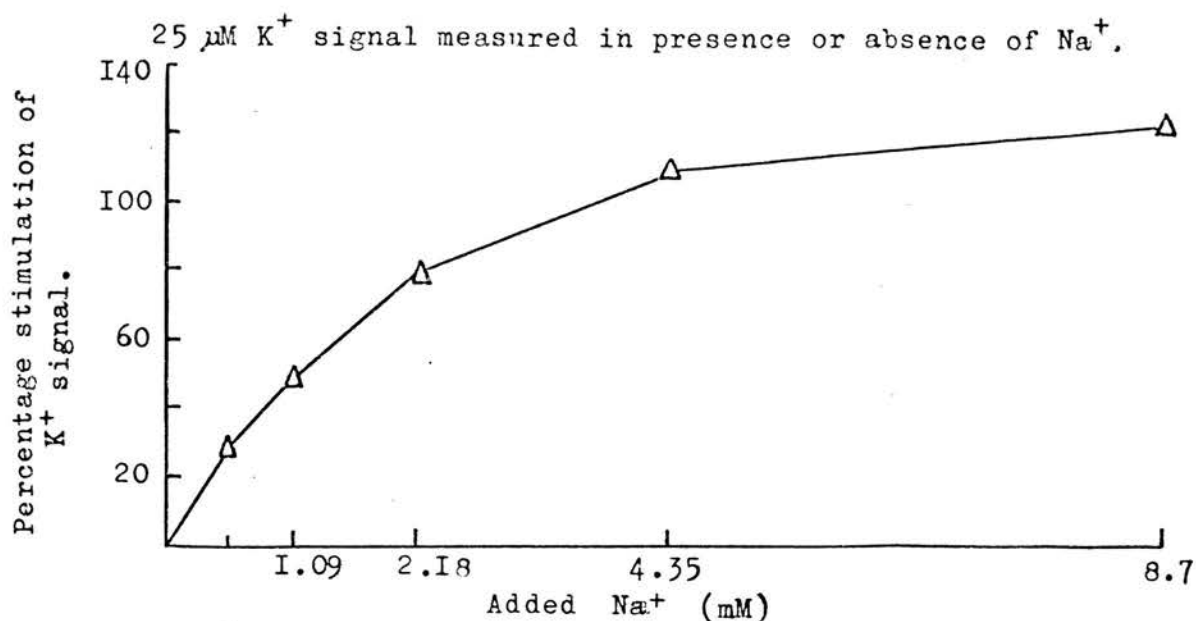
## (2) Potassium ion content

Potassium was measured by flame emission in a Perkin-Elmer 103 Atomic Absorption spectrophotometer using an air-acetylene flame. Sodium ions interfered with  $K^+$  emission and the KRB would result in errors in intracellular  $K^+$  content. Hepatocytes were washed in a sucrose buffer to remove  $Na^+$  of KRB contained between the packed cells. With variations in the quality of preparations, and possible effects of ion concentration gradients across the hepatocyte membrane, there is no certainty that the intracellular  $Na^+$  will remain low or constant. To negate possible interference from varying intracellular  $Na^+$ ,  $Na^+$  was added in excess to all samples and standards. The addition of  $Na^+$  to standard  $K^+$  solutions resulted in a stimulation over the basal  $K^+$  signal and it was maximal with 8.7mM  $Na^+$  (Fig. 3.1.A). These conditions provided a sensitive assay for  $K^+$  in the range 2-50  $\mu M$  (Fig. 3.1.B).

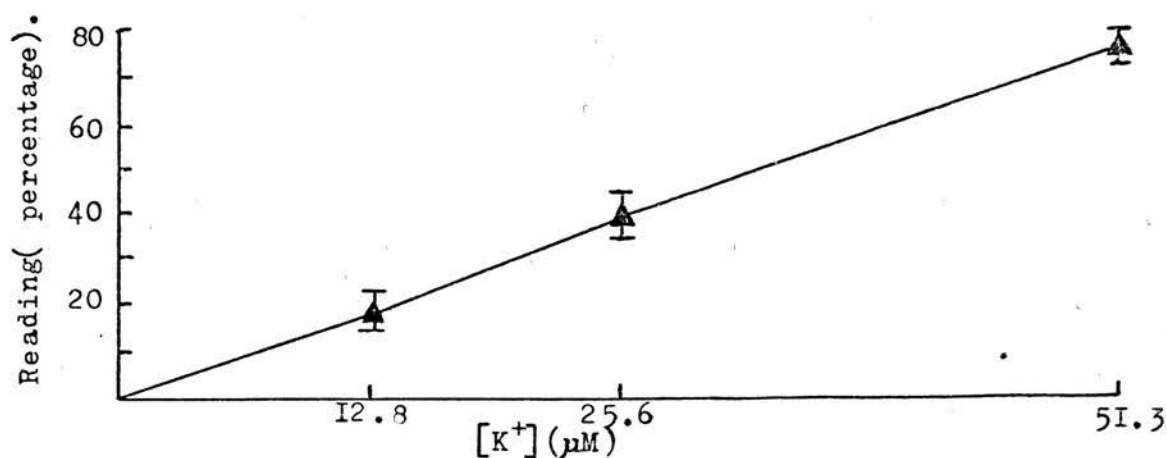
Following centrifugation (30 s at 2750 g ) of aliquots (1.5 ml ) of cell suspension, and two washes in sucrose buffer,  $K^+$  was extracted by the addition of 1 ml of 6% trichloroacetic acid to cell pellets. After 30 minutes, the samples were centrifuged (5 min at 2750 g ) and the supernatants extracted four times with ether (5 ml ). Sucrose

FIG.3.I Optimisation of  $K^+$  estimation.

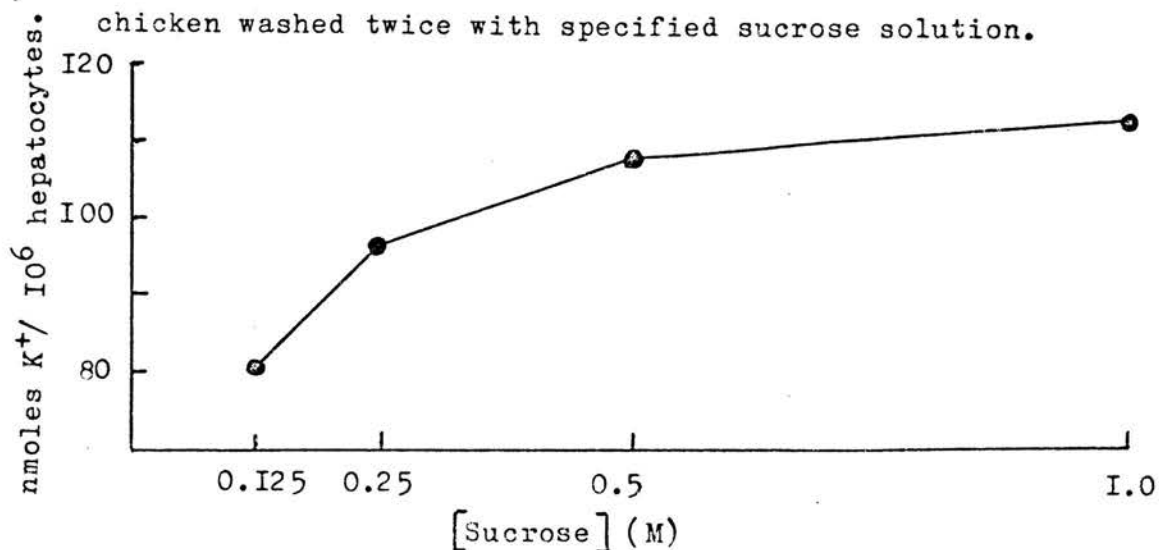
(A). Stimulatory effect of added  $Na^+$  on  $K^+$  signal.



(B).  $K^+$  standard. Each point mean  $\pm$  S.D. of 16 observations.



(C). Effect of sucrose concentration. Hepatocytes from one chicken washed twice with specified sucrose solution.



concentration influenced the  $K^+$  content and 1.0 M sucrose was used for all washes (Fig. 3.1.C).

The  $K^+$  content of pieces of liver (50-100 mg ) was measured by the same method following their homogenisation in 4 ml of ice-cold 6% trichloroacetic acid, centrifugation (2 min at 2750 g ) and extraction with ether.

All samples were assayed in quadruplicate.

### (3) Adenylate content

- (a) MEASUREMENT OF ATP. Chemiluminescent assays for ATP using luciferase are extremely sensitive and specific (Strehler, 1965). The assay of Bihler and Jeanrenaud (1970) for ATP in isolated rat fat cells was modified for isolated chicken hepatocytes.

Luciferase was prepared by incubating Firefly Lantern extract (F.L.E.) at 30°C for 15 minutes with a trace of apyrase to remove ATP contamination. The extract was made up to 0.5 mg F.L.E./ml with 50mM sodium arsenate buffer pH 7.4 containing magnesium sulphate (20 mM). It was filtered through a Whatmans No. 1 paper and stored at 4°C for two hours before use. Aliquots (2 ml) of this cocktail were pipetted into 2 x ½ inch vials supported in scintillation vials, and light emissions were recorded with a Tracerlab coincidence counter operated manually. Samples were added to the cocktail on a small plastic paddle just before the vials entered the counting chamber.

Emissions were recorded in the tritium channel and increased coarse gain significantly increased the counts due to ATP

TABLE 3.1. Optimal conditions for ATP assay.

(A) Effect of alteration of coarse gain settings on scintillation counter.

During light emission following ATP addition, counts were recorded for successive 12 s periods with alteration of coarse gain after each count.

<u>Coarse gain setting</u>	<u>Counts/12 s</u>	
	<u>Neutralised PCA (blank)</u>	<u>ATP</u>
4	2	24
8	4	467
16	3	13,566
32	6	30,144
64	4	35,435

(B) Buffer for ATP extraction.

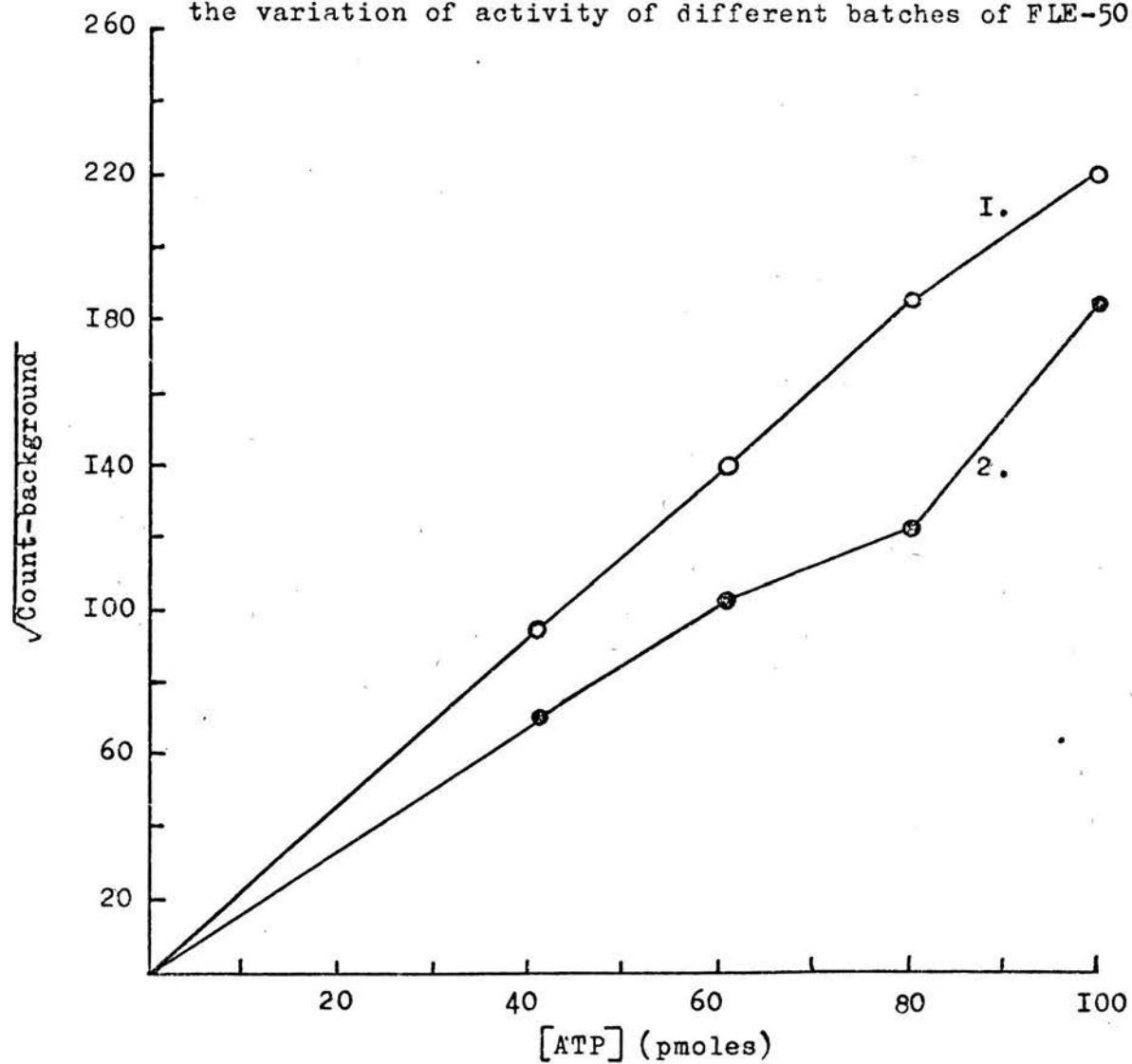
Hepatocyte adenylates extracted by pipetting cell suspensions either into ice-cold 6% PCA or 0.2M glycine/NaOH (pH 11.0) at 100°C. Number of experiments in parenthesis.

<u>Extraction</u>	<u>pmoles ATP/10<sup>6</sup> hepatocytes</u>
PCA	794.4±64.4 (7)
Glycine/NaOH	837.3±74.9 (3)



FIG. 3.2 ATP standard.

Each point is the mean of 4 observations. I and 2 illustrate the variation of activity of different batches of FLE-50.



(Table 3.1.A). Light emission is nonlinear (Strehler, 1965) and thus to achieve maximum sensitivity and reproducibility the counts were taken over the shortest possible counting time (12 s.). Under these conditions the square root of (count minus background count) was proportional to the added ATP concentration (Fig. 3.2). A full range of standards was measured for each preparation of F.L.E. as day to day variations occurred.

Bihler and Jeanrenaud (1970) extracted ATP with 0.2 M glycine (pH 11.0) at 100°C. However ATP contents were similar for isolated chicken hepatocytes whether extracted with glycine or with ice-cold 6% PCA (Table 3.1.B). All subsequent studies were carried out using PCA extraction. The extracts were either analysed the same day or stored at -50°C.

- (b) MEASUREMENT OF ADP AND AMP. These intermediates were measured by the combined assay of Adam (1965) from aliquots of the same extracts used for ATP analysis.
- (c) EFFECTS OF CENTRIFUGATION. Aliquots of cell suspensions were either added directly to PCA or rapidly centrifuged (30 s at 2750 g) and PCA subsequently added to both the pellet and supernatant fractions. Centrifugation significantly decreased the ATP, ADP and AMP contents of hepatocytes and leakage into the supernatant could not account for all the losses (Table 3.2). Similar observations have been reported for rat hepatocytes and it has been suggested that centrifugation stimulates adenylate degradation (Quistorff, et al, 1973). Thus for the estimation of adenylates aliquots of suspensions were added directly to ice-cold 6% PCA.

TABLE 3.2. Effect of centrifugation on hepatocyte adenylate content.

Ice-cold 6% PCA was added to aliquots of cell suspensions either directly or to both the pellet and supernatant fractions following centrifugation (2750g, 30 s). Results are means  $\pm$  SEM's of 3 experiments Hepatocytes were obtained from fed chickens by method A.

	<u>pmoles/10<sup>6</sup> hepatocytes</u>		<u>Percentage of total in each fraction after centrifugation</u>	
	<u>Entire suspension</u>	<u>Pelleted cells alone</u>	<u>Pellet</u>	<u>Supernatant</u>
ATP	814.6 $\pm$ 64.2	403.7 $\pm$ 76.1	97.8	2.2
ADP	532.2 $\pm$ 75.6	341.6 $\pm$ 33.2	100	ZERO
AMP	124.1 $\pm$ 56.6	79.2 $\pm$ 33.2	82.8	17.2
$\Sigma$ Adenylates	1436.2 $\pm$ 76.9	913.8 $\pm$ 70.5		
ATP/ADP	1.32 $\pm$ 0.23	1.28 $\pm$ 0.24		

- (d) **ADENYLATE CONTENT OF WHOLE LIVER.** To extract adenylates from whole liver, chickens were killed by cervical dislocation, the liver rapidly clamped in precooled aluminium tongs and immersed in liquid  $N_2$  within 5 to 12 seconds of dislocation. Pieces of liver were then powdered in a precooled porcelain mortar with repeated additions of liquid  $N_2$ . While chilled, the powder was homogenised in ice-cold 6% PCA and then centrifuged for 4 minutes at 2800 g ( $4^{\circ}C$ ). The pellet was re-extracted with ice-cold 3% PCA and re-centrifuged. The pooled supernatants were neutralised with  $K_2CO_3$  (3M), and the  $KClO_4$  formed was removed by centrifugation.

Slices prepared from liver immediately following the perfusion stage of cell preparation were homogenised in ice-cold 6% PCA. Further extraction of adenylates was carried out as described above.

(4) Oxygen consumption

This was measured with a Clark-type oxygen electrode. Cell suspensions (2 ml) and KRB (2 ml) equilibrated at  $40^{\circ}C$  were added to the water-jacketed chamber ( $40^{\circ}C$ ) of the oxygen electrode and stirred continuously by a small magnetic flea. The change in oxygen content was represented on a Heathkit recorder. Increased oxygen consumption of submitochondrial particles from chicken hearts due to NADH addition enabled the electrode to be calibrated (Estabrook, 1967). The oxygen content of gassed KRB was calculated to be 1.215 mM. This compares favourably with the theoretical value of 1.018 mM for KRB at  $40^{\circ}C$  calculated from a Bunsen Coefficient ( $\alpha$ ) of 0.023 for dilute salt solutions at  $40^{\circ}C$  (Dawson, et al, 1969).

Hepatocytes consumed oxygen at a constant rate for 30 minutes. Leakage from the electrode made it necessary to measure the blank rate of oxygen loss before each determination. Aliquots (0.2 ml ) withdrawn from the chamber for light microscopic examination permitted quantification of results and showed that the magnetic stirrer did not damage the hepatocytes.

Oxygen consumption of suspensions was measured with endogenous substrates and also after the addition of 10  $\mu$ l aliquots of substrates to give a final concentration of 10 mM.

#### (5) Glycogen content

Hepatocyte suspensions (1.5 ml ) were centrifuged (30 s at 2750 g ), aliquots of the supernatant assayed for glucose and the pellet digested with 30% KOH. Glycogen was precipitated with 2%  $\text{Na}_2\text{SO}_4$ /absolute ethanol at 4°C overnight and the precipitates were washed twice with 70% ethanol. Following hydrolysis with 1M  $\text{H}_2\text{SO}_4$  (1 ml ), 0.1 M sodium phosphate buffer, pH 7.0 (1 ml ) and 2M NaOH (1 ml ) were added to neutralise the digest and the glycogen was estimated as glucose equivalents using glucose oxidase (Hugget & Nixon, 1957). A stock reagent mixture was made up in 0.1 M-phosphate buffer (pH 7.0) containing 4-aminophenazone (0.3 mg/ml), phenol (1.0 mg/ml), glucose oxidase (9 mg/100 ml) and peroxidase (3 mg/100 ml). Aliquots (2 ml) of this reagent were added to 0.1 ml of samples and the colour developed over 40 minutes at 40°C and read at 510 nm.

Precipitates of hepatocytes from starved chickens were digested in 0.5 ml  $\text{H}_2\text{SO}_4$  and the digests made up to a final volume of 1.5 ml. With this modification and the use of 0.5 ml of samples in the assays, the glycogen content of starved chicken hepatocytes could be measured.

#### (6) Dry weight, protein and DNA content

These characteristics were measured for quadruplicate 1 ml samples of cell suspensions. Protein was measured by the method of Lowry, et al (1951) in 10% trichloroacetic acid-insoluble extracts of cell pellets

TABLE 3.3. Metabolic characteristics of chicken hepatocytes after isolation.

All hepatocytes were isolated with 0.25 mg collagenase/ml, and 1.0 mg hyaluronidase/ml was also included as indicated. The nutritional state of chickens from which cells are isolated is indicated (F - Fed; S - 24h. starved). Values expressed as units /10<sup>6</sup> hepatocytes by means  $\pm$  SEM's. Number of experiments in parentheses.

<u>Characteristic</u>	<u>Nutritional State</u>	<u>Hyaluronidase</u>	<u>Value</u>
Lactate dehydrogenase (mU) activity	F	-	251 $\pm$ 30 (10)
Glutamate dehydrogenase activity (mU)	F	-	104 $\pm$ 19 (5)
Aspartate aminotransferase activity (mU)	F	-	207 $\pm$ 25 (5)
Potassium ion content (nmoles)	F	-	75.42 $\pm$ 9.10 (6)
"	F	+	66.95 $\pm$ 3.89 (6)
Oxygen consumption ( $\mu$ ls/h)	F	-	8.09 $\pm$ 1.05 (8)
"	F	+	10.21 $\pm$ 1.02 (8)
"	S	-	8.60 $\pm$ 1.11 (3)
"	S	+	7.35 $\pm$ 1.85 (3)
Glycogen content ( $\mu$ moles glucose equivalent)	F	-	37.90 $\pm$ 2.08 (20)
"	S	-	1.21 $\pm$ 0.56 (6)
DNA content ( $\mu$ g)	F	-	5.89 $\pm$ 0.42 (19)
Protein content ( $\mu$ g)	F	-	122.1 $\pm$ 13.3 (20)
Dry weight (mg)	F	-	0.340 $\pm$ 0.045 (13)

Students t-tests indicated that hyaluronidase did not significantly influence potassium ion content or oxygen consumption.

TABLE 3.4. Adenylate content of hepatocytes immediately after isolation.

Hepatocytes isolated with 0.25 mg collagenase/ml by either method A or B as stated. Values are expressed as pmoles/ $10^6$  hepatocytes and are means  $\pm$  SEM's. The number of experiments are given in parentheses.

	<u>METHOD A</u>		<u>METHOD B</u>
	<u>Fed chicken</u>	<u>24h starved chicken</u>	<u>Fed chicken</u>
ATP	794.4 $\pm$ 64.4*	935.8 $\pm$ 36.3*	972.1 $\pm$ 67.4
ADP	425.2 $\pm$ 107.8	410.7 $\pm$ 105.6	1458.1 $\pm$ 205.0
AMP	90.3 $\pm$ 40.5	142.0 $\pm$ 36.9	1353.9 $\pm$ 182.4
$\Sigma$ Adenylates	1207.8 $\pm$ 169.2	1488.5 $\pm$ 105.9	3795.2 $\pm$ 258.1
ATP/ADP	1.94 $\pm$ 0.22	2.28 $\pm$ 0.55	0.73 $\pm$ 0.15
	(5)	(6)	(5)

\* Student t-test:  $P < 0.025$

after centrifugation (30 s at 2750 g) and two washes in KRB without BSA. DNA was measured in 6% PCA extracts of cell pellets by the diphenylamine reaction of Burton (1956).

Dry weight was calculated by drying aliquots to constant weight at 120°C and subtracting the dry weight of KRB containing 2% BSA. Aliquots of liver homogenates were added to TCA and heated to dryness. A wet to dry weight ratio of  $3.74 \pm 0.21$  (3) was obtained for chicken liver. This ratio has been used to obtain wet weights of hepatocytes for comparison to those of whole liver (Krebs, et al, 1974).

These characteristics are expressed as units per  $10^6$  hepatocytes. For expressing the changes in metabolic activity during incubation, the initial value is termed as 100% and other values expressed as percentages of the initial.

## RESULTS

### Characteristics of hepatocytes immediately after isolation

The  $K^+$  content of hepatocytes isolated from fed chickens was similar whether hyaluronidase was present or absent during preparation (Table 3.3). Whole liver contained  $76.9 \pm 3.8$   $\mu$  mole  $K^+$ /g and cells  $59.3 \pm 7.2$   $\mu$  moles/g.

Total adenylate contents of hepatocytes prepared by methods A and B were markedly different (Table 3.4). Method B gave total adenylate contents ( $3.79$   $n$ .moles/ $10^6$  hepatocytes) three times that of method A ( $1.21$   $n$ .moles/ $10^6$  hepatocytes). This difference was due almost entirely to retention of ADP and AMP. Values obtained by method B expressed per g wet weight of hepatocytes give a value ( $2.98$   $\mu$  moles/g.) similar to



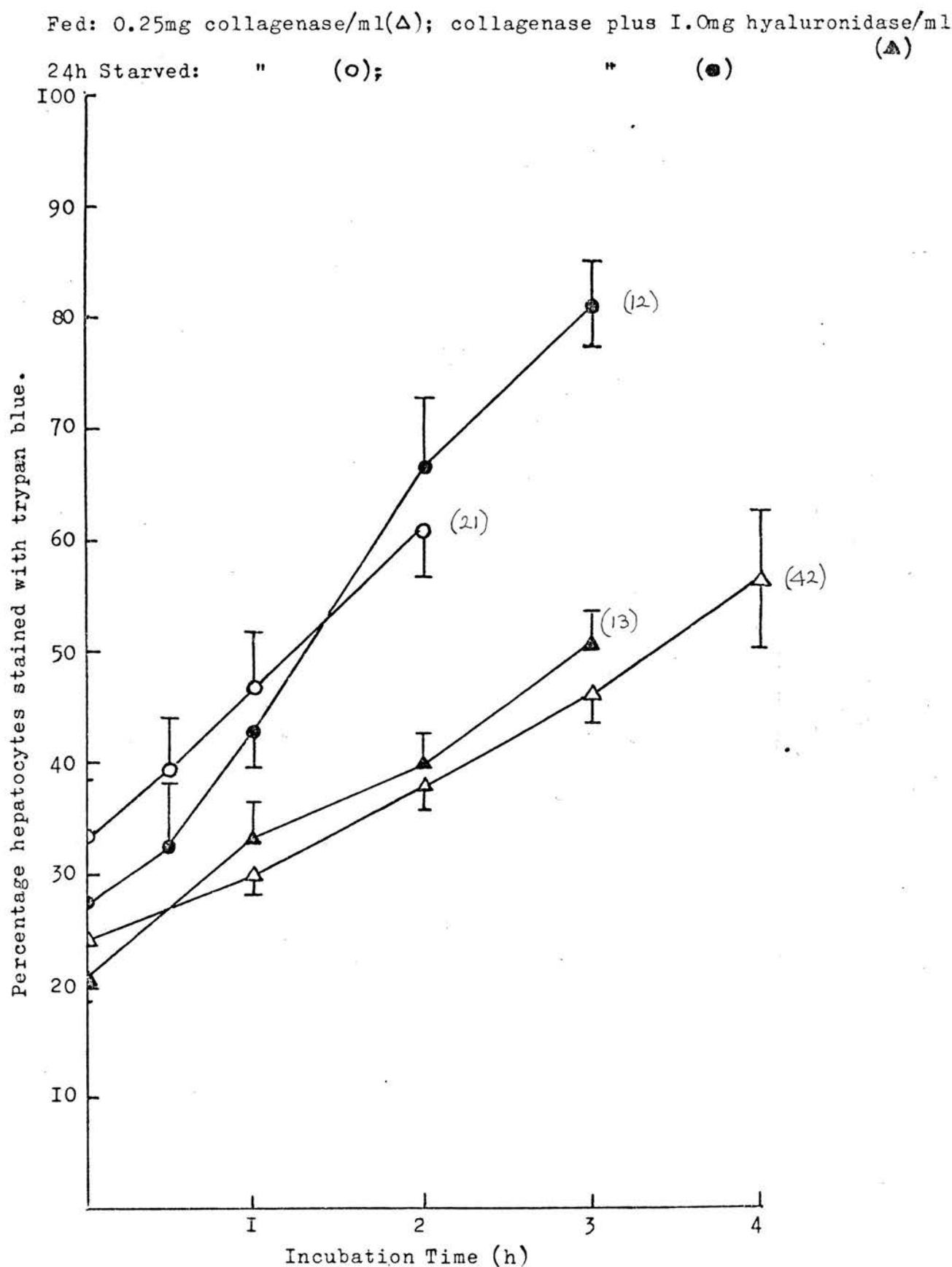
TABLE 3.5. Adenylate content of freeze-clamped chicken liver and liver slices.

Values are  $\mu\text{moles/g}$  wet weight and are expressed as means  $\pm$  SEM's. The number of experiments are given in parentheses.

<u>SAMPLE</u>	<u>ATP</u>	<u>ADP</u>	<u>AMP</u>	<u><math>\Sigma</math>Adenylates</u>	<u>ATP/ADP</u>
Whole liver:					
Fed chicken (14)	0.32* $\pm 0.03$	1.34 $\pm 0.08$	0.81 $\pm 0.04$	2.46** $\pm 0.12$	0.24 $\pm 0.02$
24h starved chicken (14)	0.38* $\pm 0.03$	1.48 $\pm 0.07$	1.02 $\pm 0.10$	2.87** $\pm 0.13$	0.27 $\pm 0.03$
Liver slices:					
Fed chicken (3)	0.45 $\pm 0.10$	0.71 $\pm 0.14$	0.19 $\pm 0.17$	1.64 $\pm 0.17$	0.52 $\pm 0.18$

Student t-test: \*  $P < 0.0025$   
 \*\*  $P < 0.0005$

FIG. 3.3 Effect of feeding state and enzyme conditions used for isolation on percentage increase of trypan blue stained hepatocytes during incubation.



Values are means  $\pm$  SEM for the number of experiments indicated in brackets.

Hepatocytes were obtained by method A.

FIG. 3.4 Effect of isolation method on trypan blue  
staining.

Hepatocytes isolated from fed chickens with 0.25 mg collagenase/ml  
by method A (●) or B (Δ). (30 and 5 experiments respectively)

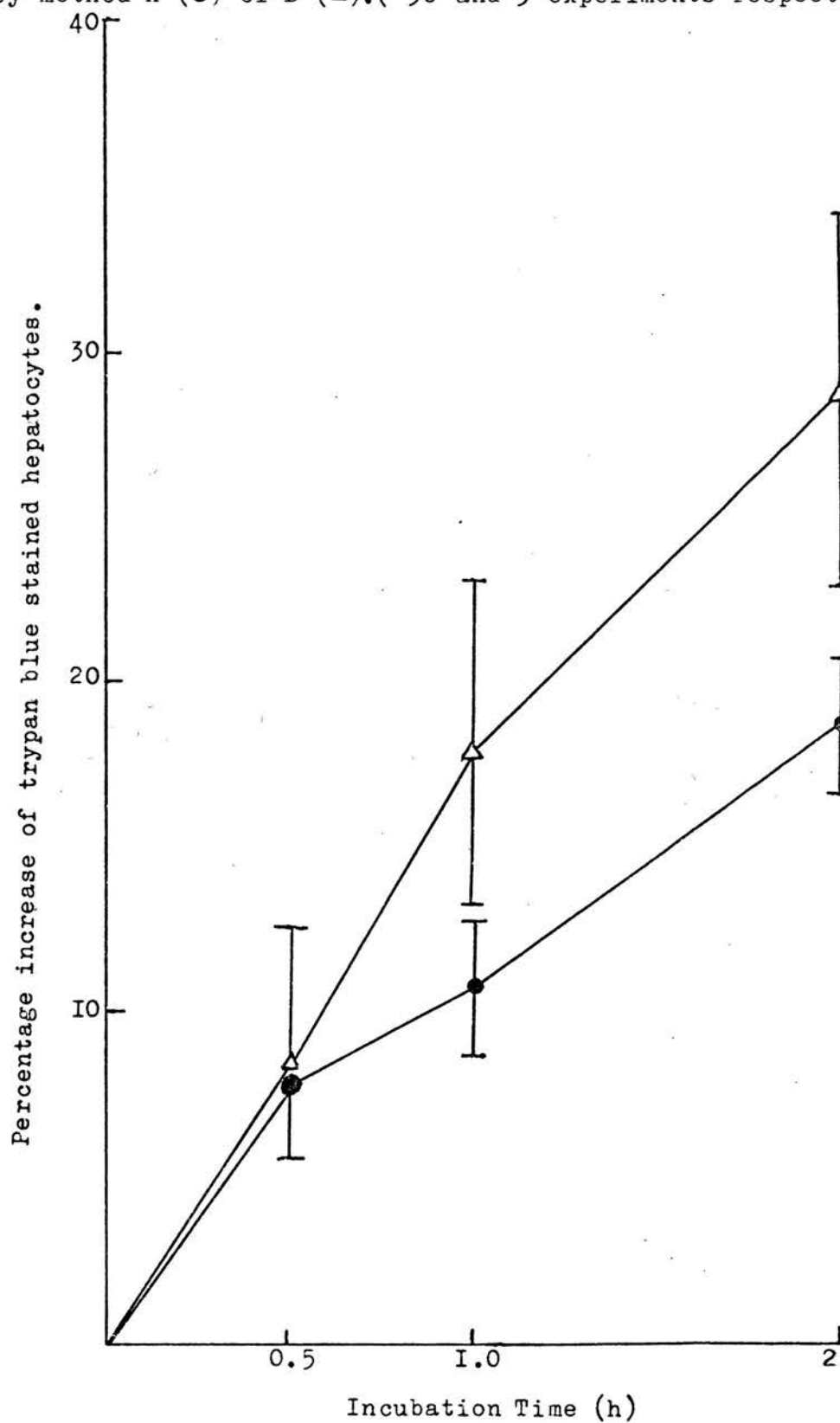


FIG. 3.5 Leakage of enzymes during incubation.

Hepatocytes isolated from fed chickens with 0.25mg collagenase/ml only by method A.  
 $\Delta$  Lactate dehydrogenase;  $\blacktriangle$  Glutamate dehydrogenase;  $\circ$  Aspartate

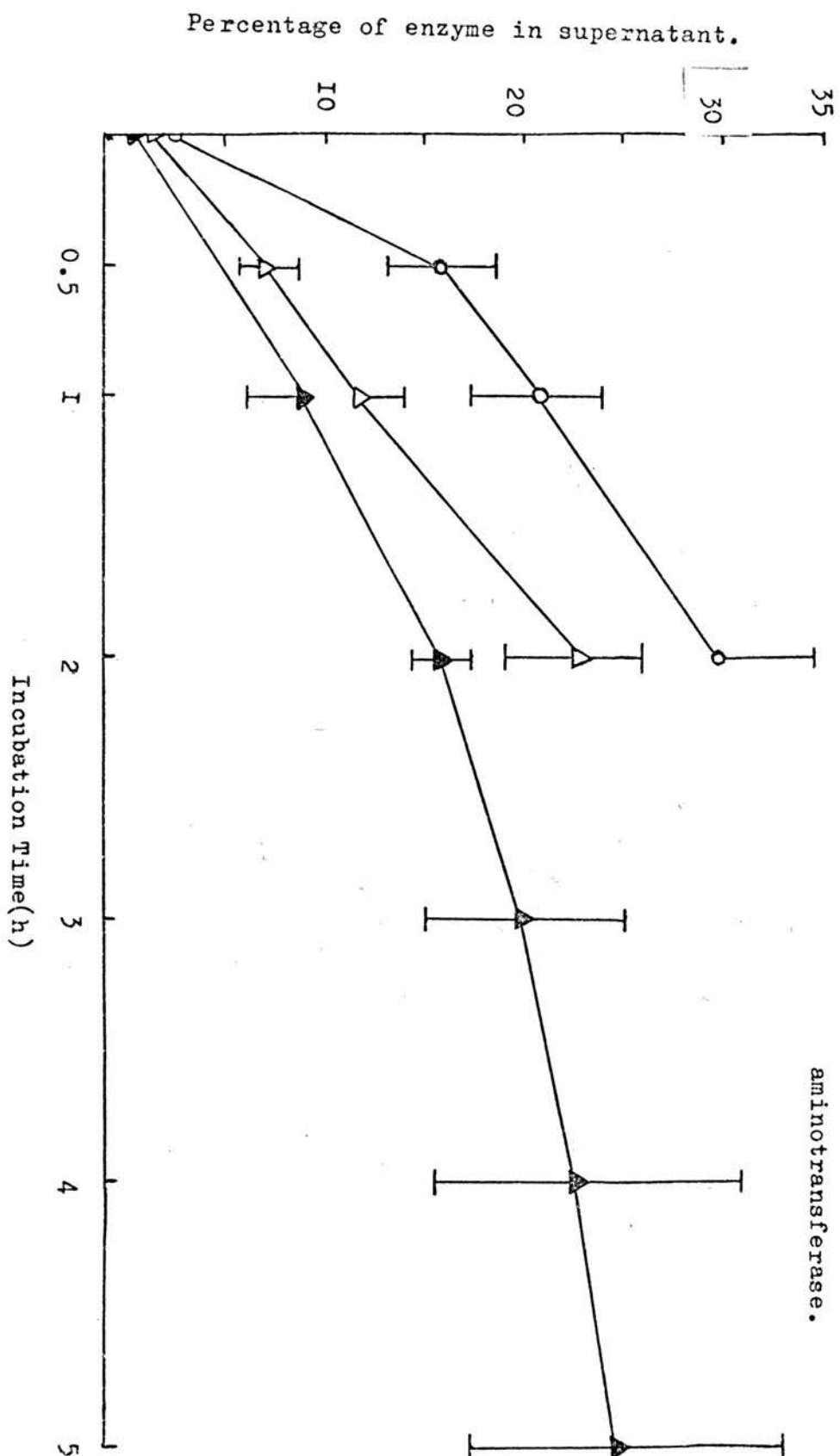


FIG. 3.6 Linear regression analysis of the percentage increase in trypan blue

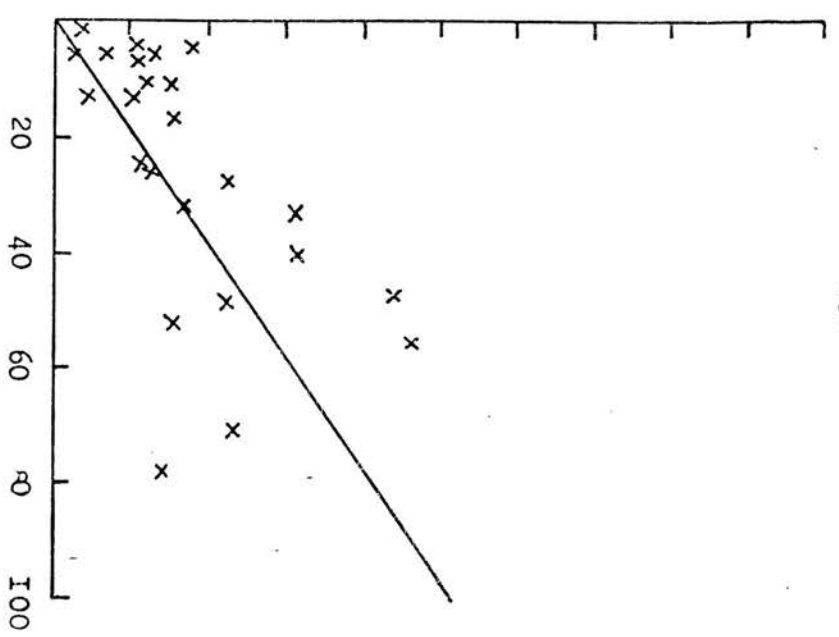
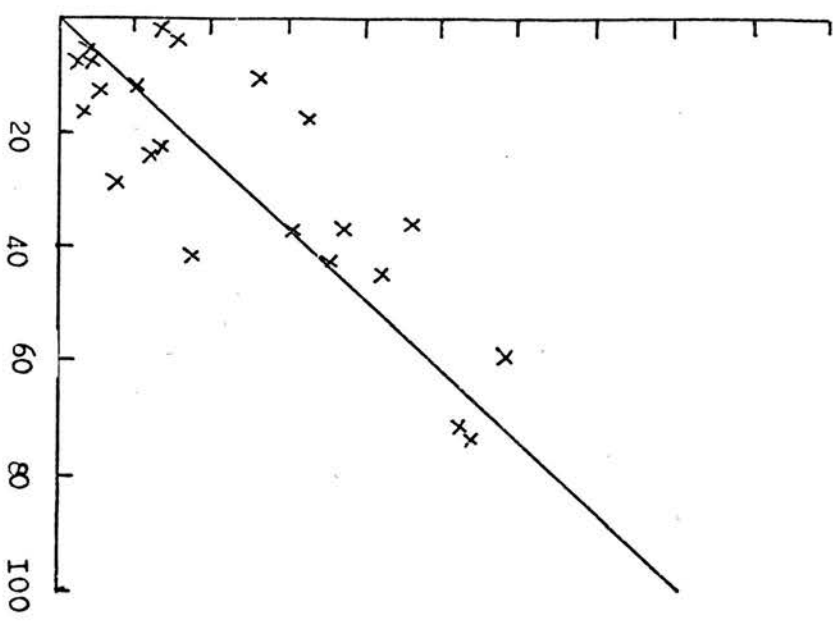
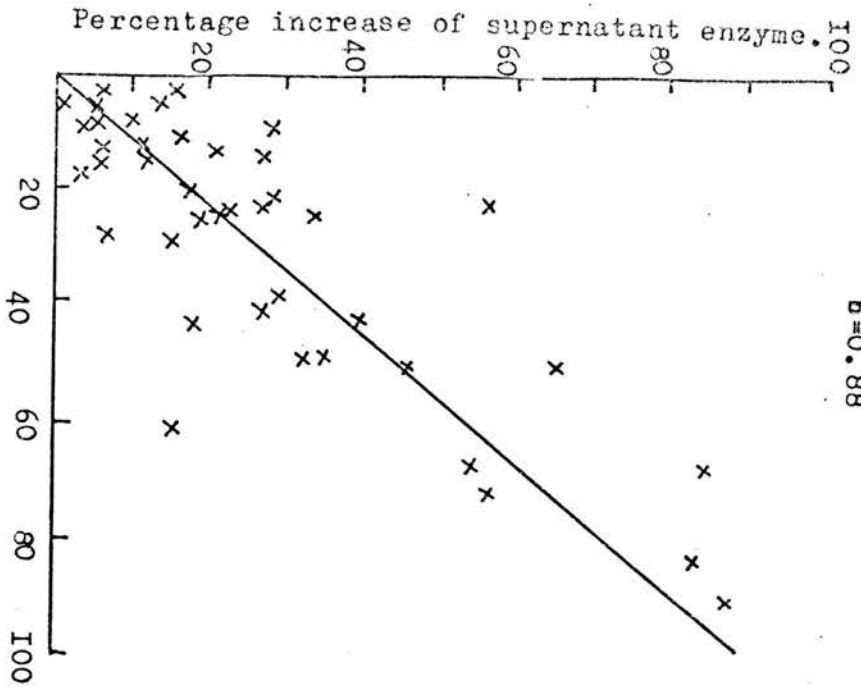
stained hepatocytes and the percentage increase of enzymes in the  
supernatant fraction.

Correlation coefficients(b) obtained by data fitting with Olivetti desktop programmable calculator.

Lactate dehydrogenase.  
 $b=0.88$

Aspartate aminotransferase.  
 $b=0.80$

Glutamate dehydrogenase.  
 $b=0.51$



Percentage increase of trypan blue stained hepatocytes.

that of whole liver of 2.46  $\mu$  moles/g. (Table 3.5). The ATP and total adenylate contents for freeze-clamped liver from fed and 24 hour starved chickens were significantly different (Table 3.5) and to a smaller extent this was found for hepatocytes isolated by method A (Table 3.4). Whether this is of physiological significance is unknown.

Endogenous oxygen consumption was similar for hepatocytes isolated from fed and 24 hour starved chickens and hyaluronidase presence during isolation had no significant effect on oxygen consumption (Table 3.3). Consumption was stimulated by the addition of neutralised lactate to hepatocytes from both fed ( $40.6 \pm 7.1\%$  stimulation) and 24 hour starved chickens ( $25.7 \pm 8.2\%$ ).

Starvation of chickens for 24 hours prior to hepatocyte isolation decreased the glycogen content of hepatocytes (Table 3.3).

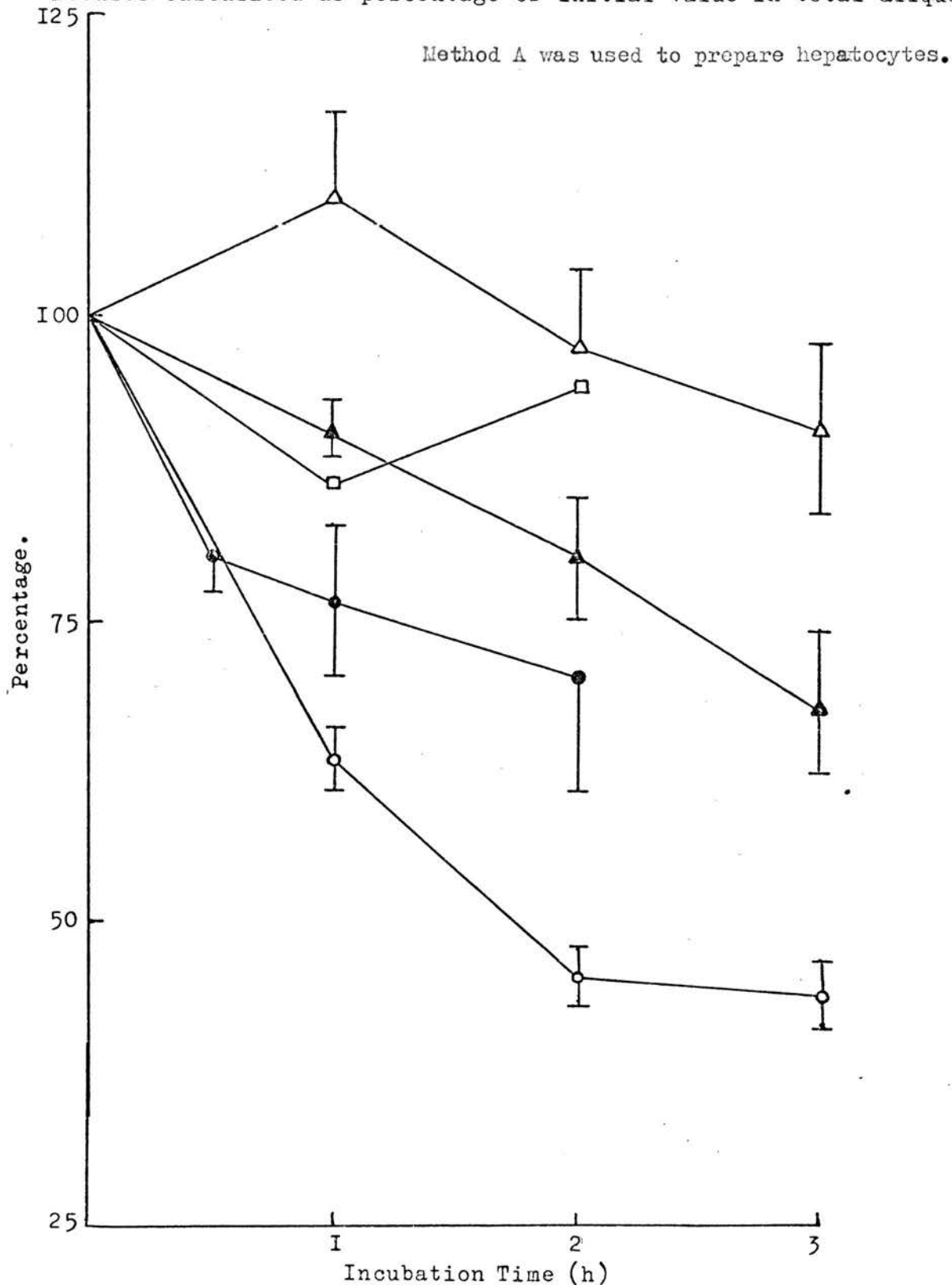
#### Effects of incubation

Incubation at 40°C resulted in a progressive increase in the percentage of stained hepatocytes and was greater for hepatocytes isolated from starved chickens than from fed (Fig. 3.3). This increase was unaffected by the presence or absence of hyaluronidase during isolation and occurred for cells isolated by methods A or B (Fig. 3.4). The proportion of intracellular enzymes in the supernatant was low initially due to thorough washing during isolation and increased throughout incubation (Fig. 3.5). The percentage increases in the leakage of all three enzymes and trypan blue staining was correlated (Fig. 3.6).

Since there was a progressive increase of trypan blue stained hepatocytes, there is no certainty that either the metabolic activity

FIG. 3.7 Alterations in staining,  $K^+$ , ATP and adenylate content and oxygen consumption with incubation at 40°C.

Fed chicken hepatocytes isolated with 0.25mg collagenase/ml and results calculated as percentage of initial value in total aliquot.



$\Delta K^+$  content(6)

● ATP content(10)

$\blacktriangle$  Trypan blue exclusion(19)

□ Adenylate content(9)

○ Oxygen consumption(8)

Number of experiments in parentheses. Values are means  $\pm$  SEM.

TABLE 3.6. Effect of incubation on decreases in endogenous oxygen consumption and K<sup>+</sup> content.

Hepatocytes were isolated from fed (F) or 24h starved (S) chickens with 0.25 mg collagenase/ml, and, where indicated, 1.0 mg hyaluronidase/ml. Results are expressed as percentages of initial values (Table 3.3.) and are means  $\pm$  SEM's. The number of experiments are given in parentheses. Hepatocytes were prepared by method A.

<u>CHARACTERISTIC</u>	<u>INCUBATION(h)</u>	<u>NUTRITIONAL STATE OF CHICKEN:</u>		
		F	F	S
		Hyaluronidase present:		
		-	+	-
Oxygen consumption (4)	1	63.5 $\pm$ 2.4	54.8 $\pm$ 0.9	54.3 $\pm$ 7.5
	2	45.8 $\pm$ 2.1	43.0 $\pm$ 2.2	39.5 $\pm$ 4.8
	3	44.4 $\pm$ 2.8	31.5 $\pm$ 3.3	25.9 $\pm$ 3.9
K <sup>+</sup> content (6)	1	110.4 $\pm$ 6.7	101.7 $\pm$ 5.6	
	2	98.3 $\pm$ 5.5	81.0 $\pm$ 6.5	
	3	91.1 $\pm$ 6.9	79.0 $\pm$ 8.4	



TABLE 3.7. Effect of incubation on ATP/ADP ratio.

Hepatocytes isolated by method A from fed chickens with 0.25 mg collagenase/ml only. Mean of 5 experiments  $\pm$  SEM.

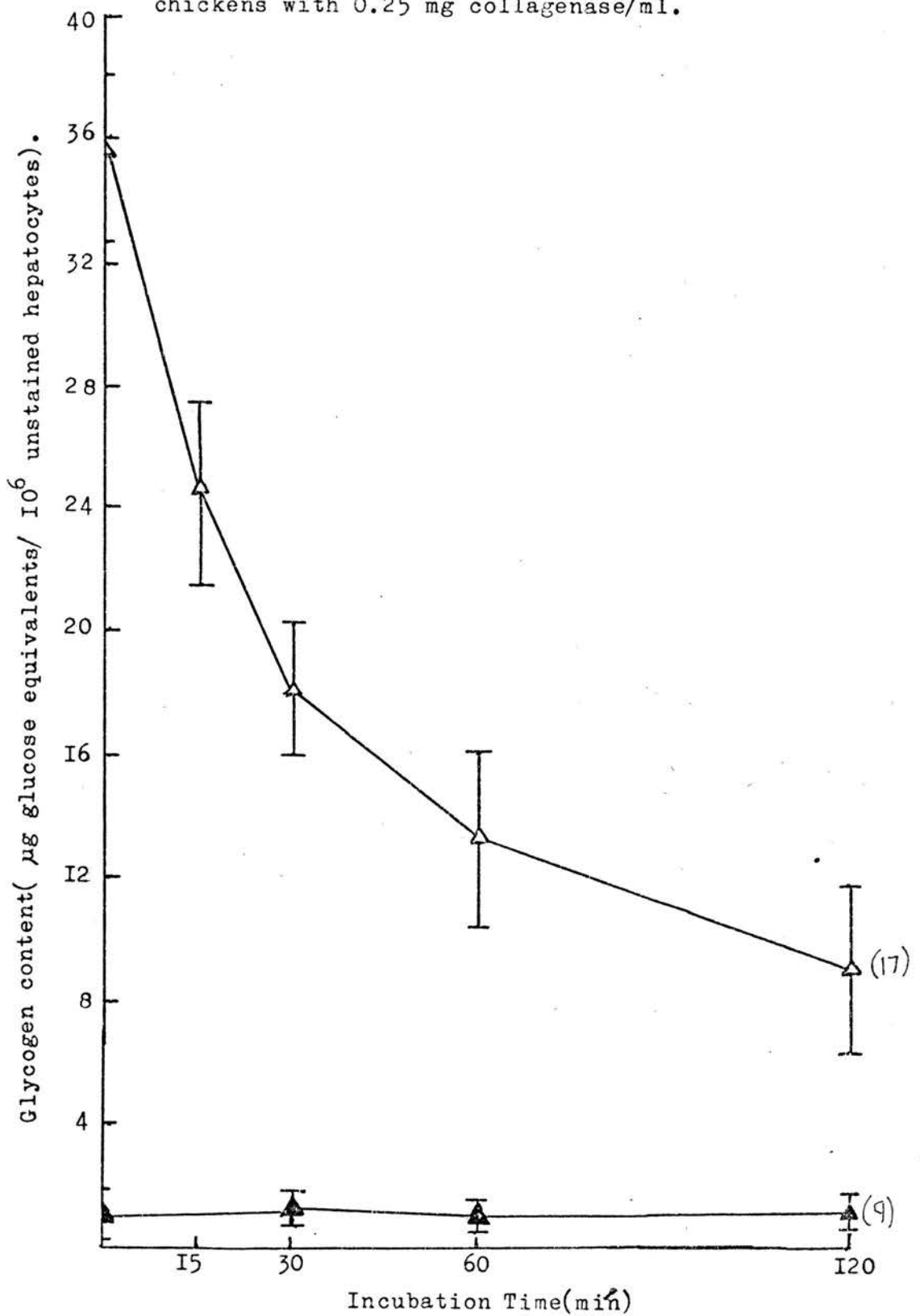
<u>Time of incubation</u>	<u>ATP/ADP</u>
*0	2.40 $\pm$ 0.32
1	1.71 $\pm$ 0.26
*2	1.72 $\pm$ 0.18

\* Student t-test between 0 and 2 values.  $P < 0.1$

FIG. 3.8 Change in hepatocyte glycogen content during incubation

Hepatocytes isolated from fed( $\Delta$ ) and 24h starved( $\blacktriangle$ )

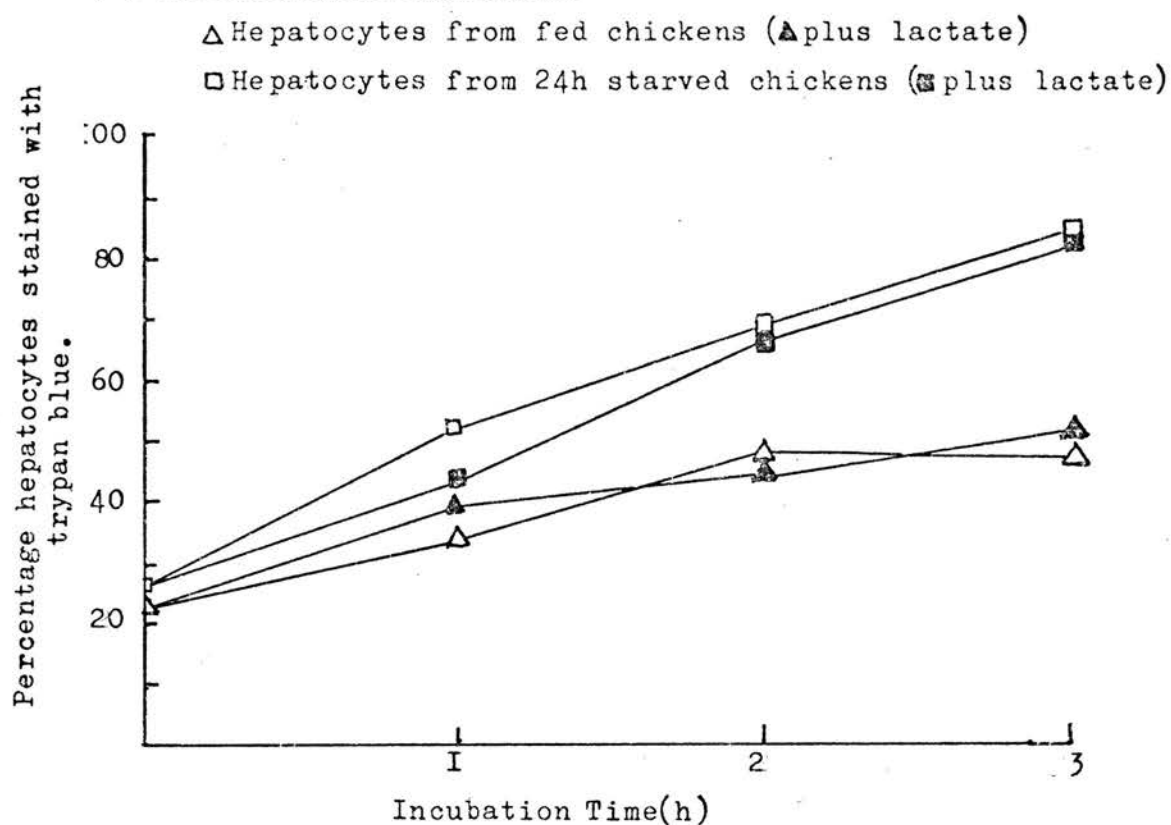
chickens with 0.25 mg collagenase/ml.



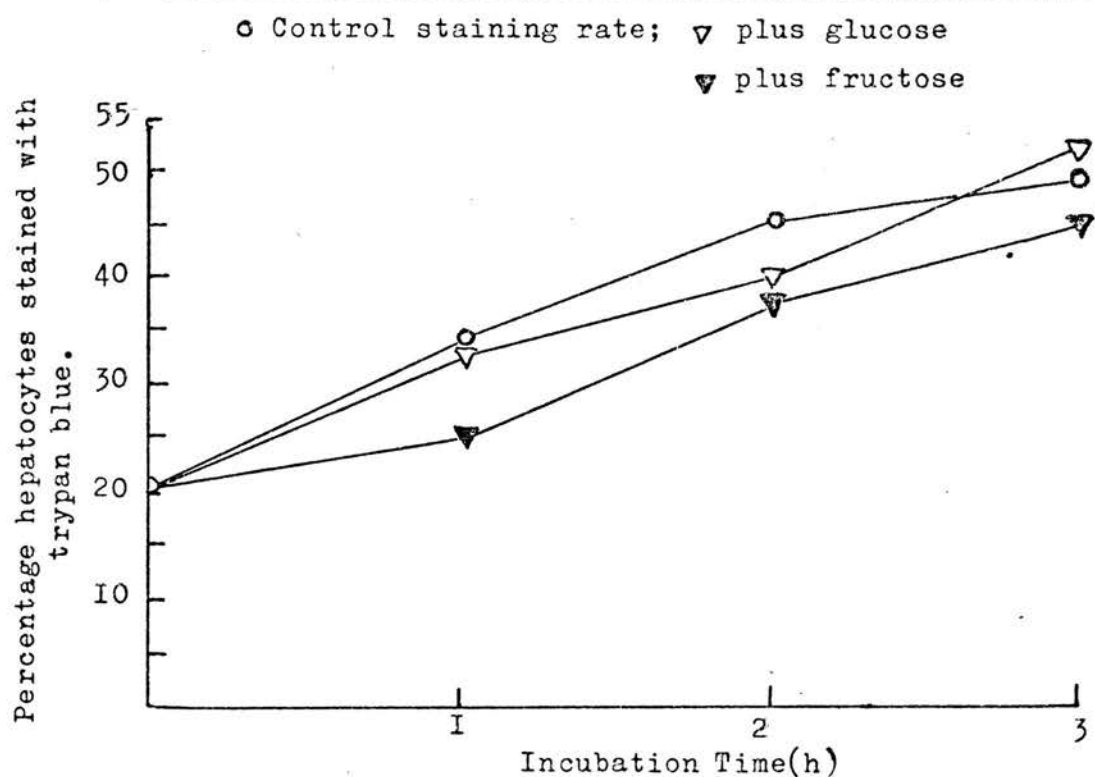
Hepatocytes were prepared by method B and points are means  $\pm$  SEM for the number of experiments given in brackets.

FIG. 3.9 Effects of substrate addition on trypan blue staining.

(A). Effects of 10mM lactate.



(B). Effects of 10mM glucose and fructose on fed chicken hepatocytes



Results were obtained from single experiments with hepatocytes obtained by method A.

TABLE 3.8. Effects of substrate on endogenous oxygen consumption.

Hepatocytes were isolated with 0.25 mg collagenase/ml only and results are mean percentage stimulations of oxygen consumption  $\pm$  SEM's. The number of experiments are given in parentheses.

Hepatocytes were prepared by method A.

A. Hepatocytes were incubated in the oxygen electrode chamber when 10  $\mu$ l of neutralised lactate was added to give a final concentration of 10 mM. The percentage stimulation of oxygen uptake was noted at various times of incubation.

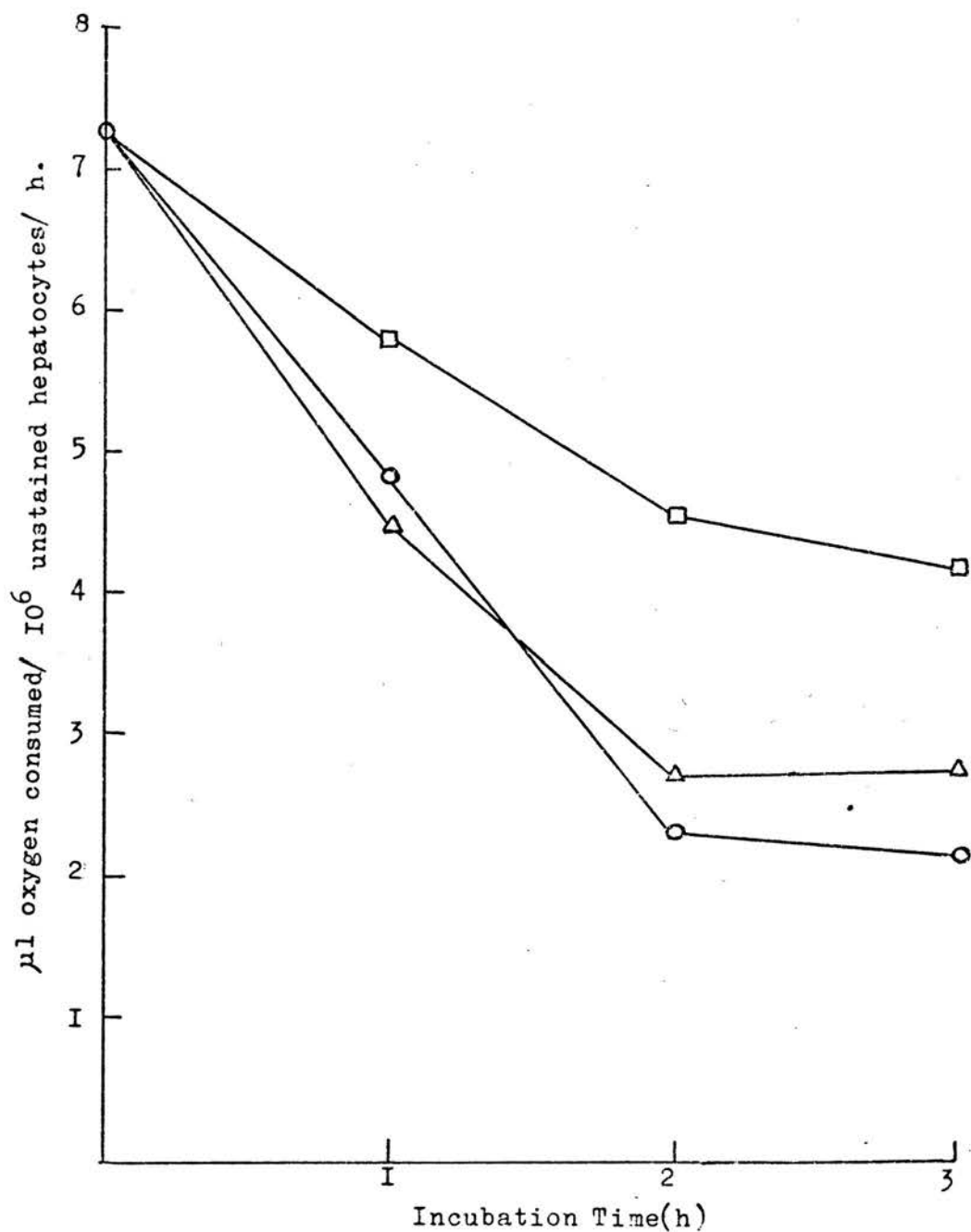
<u>Nutritional state of chicken</u>	<u>Incubation Time (h)</u>			
	<u>0</u>	<u>1</u>	<u>2</u>	<u>3</u>
Fed (7)	40.6 $\pm$ 7.1	54.3 $\pm$ 10.8	51.2 $\pm$ 14.0	70.5 $\pm$ 18.6
24h starved (4)	25.7 $\pm$ 8.2	37.8 $\pm$ 1.4	31.1 $\pm$ 7.1	32.7 $\pm$ 3.2

B. Hepatocytes from fed chickens were incubated in incubation pots with and without substrates and aliquots were withdrawn at specified times for analysis of oxygen consumption. Values were obtained by comparison to the control sample and represent percentage stimulation.

<u>Substrate added</u>	<u>Incubation Time (h)</u>		
	<u>1</u>	<u>2</u>	<u>3</u>
10 mM Glucose (3)	1.0 $\pm$ 7.4	34.4 $\pm$ 2.7	44.0 $\pm$ 7.1
10 mM Fructose (3)	31.9 $\pm$ 6.5	156.2 $\pm$ 12.3	150.9 $\pm$ 1.6

FIG. 3.10 Effects of fructose and glucose on oxygen consumption  
during incubation.

Hepatocytes isolated from fed chickens incubated alone(O) or with 10mM fructose( $\square$ ) or glucose( $\Delta$ ). Samples withdrawn at specified times for analysis of rate of oxygen consumption.



Values are the means of two experiments with cells prepared by method A.

TABLE 3.9.      Sustrate effects on ATP and adenylate contents of hepatocytes.

Hepatocytes were isolated from fed chickens with 0.25 mg collagenase/ml only. ATP and adenylate contents are expressed as pmoles/ $10^6$  hepatocytes and are means  $\pm$  SEM's.

A.    Effect of Lactate

Hepatocytes isolated by method A were incubated for 60 minutes in the presence or absence of lactate (10mM) before adenylate analysis. The means of two experiments are given below.

	<u>CONTROL</u>	<u>PLUS LACTATE</u>
ATP	746.2	661.8
$\Sigma$ Adenylates	1160.4	1394.9
ATP/ADP	2.28	0.97

B.    Effect of fructose

Hepatocytes isolated by method B were incubated for 60 minutes in the presence or absence of fructose (10mM). Values below are the means of 4 experiments  $\pm$  SEM's.

	<u>CONTROL</u>	<u>PLUS FRUCTOSE</u>
ATP	1256.5 $\pm$ 61.7	1092.5 $\pm$ 62.1
$\Sigma$ Adenylates	4543.5 $\pm$ 120.4	6075.8 $\pm$ 149.8
ATP/ADP	0.88 $\pm$ 0.27	0.42 $\pm$ 0.04

of the total suspension remains constant or of the contribution of stained cells to metabolic activities. Basal oxygen consumption,  $K^+$ , ATP and adenylate contents of suspensions decreased during incubation (Fig. 3.7) and were unaffected by the presence of hyaluronidase during isolation (Table 3.6). The ATP/ADP ratio fell during the first hour of incubation but then remained constant over the next hour (Table 3.7). The low initial ATP/ADP ratio of hepatocytes isolated by method B remained constant during incubations.

The glycogen content of fed chicken hepatocytes decreased rapidly over the first hour of incubation, whereas the glycogen content of hepatocytes from 24 hour starved chickens did not change significantly from the initial low contents (Fig. 3.8).

So far all results described are for hepatocytes incubated without substrates. Hepatocytes in situ are surrounded by exogenous substrates and hence cells may require added substrate to maintain their integrity. This was investigated by incubating hepatocytes with glucose, fructose and lactate. Added substrates had no effect on the rate of trypan blue staining of hepatocytes (Fig. 3.9). Oxygen consumption was stimulated throughout the three hour incubation period but this stimulation was greatest between the second and third hours of incubation (Table 3.8). As endogenous oxygen consumption fell over the first hour of incubation before reaching a plateau between the second and third hours (Fig. 3.7), substrate additions maintained oxygen consumption nearer the initial value (Fig. 3.10). Incubation of hepatocytes with lactate resulted

in a lowering of ATP content and ATP/ADP ratio, although the total adenylate content was maintained (Table 3.9A). Similar effects were found during incubation with fructose (Table 3.9B) in contrast

to the dramatic falls in hepatic ATP and adenylate contents reported for rat liver in vitro (Woods, et al, 1970) and in vivo (Chagoy de Sanchez & Pina, 1972).

## DISCUSSION

### Quality of preparations

One method of assessing the quality of isolated hepatocytes is to compare their characteristics to those of whole liver. Many workers compare their results in terms of units per gram of wet weight but the isolation of different cell types from liver indicates that functional variations occur (Crisp & Pogson, 1972; Quistorff, et al, 1973). Thus one gram wet weight of isolated hepatocytes may not be synonymous with one gram wet weight of liver. The use of other physical methods for comparison such as dry weight, protein and DNA content have similar limitations. Essentially, the only method for exact comparison would be to use a specific parenchymal cell marker enzyme as the unit of activity. However for routine comparisons wet weight may suffice. Using the conversion factor for wet to dry weight (3.74) found for whole chicken liver, one gram of parenchymal cells contains  $4.63 \pm 0.57$  mg DNA/g wet weight. Whole liver contains  $4.67 \pm 0.57$  mg DNA/g wet weight (7 observations). The similarity of these values suggests that the use of this conversion factor is justified. Using this basis, the metabolic characteristics of whole chicken liver, isolated chicken and rat hepatocytes can be compared (Table 3.10).

The procedures employed during isolation result in no significant alteration of enzyme activities (Table 3.10). Although the extracellular space of whole liver has not been taken into account for the  $K^+$  content,



TABLE 3.10. Comparison of metabolic characteristics of whole chicken liver, isolated chicken and rat hepatocytes.

Expressed as units/g wet weight. Sources of rat hepatocytes data are given below, and except where specifically mentioned all chicken values were obtained in the present study.

	<u>CHICKEN</u> <u>LIVER</u>	<u>CHICKEN</u> <u>HEPATOCYTES</u>	<u>RAT</u> <u>HEPATOCYTES</u>
Lactate dehydrogenase(U)	206.6 $\pm$ 15.5 <sup>1</sup>	197.3 $\pm$ 23.6	162.1 <sup>8</sup>
Glutamate dehydrogenase(U)	36.6 $\pm$ 3.6	82.5 $\pm$ 14.7	32.4 <sup>8</sup>
Aspartate aminotransferase(U)	90.6 $\pm$ 12.0	162.5 $\pm$ 19.6	130.2 <sup>8</sup>
K <sup>+</sup> content( $\mu$ .moles)	76.9 $\pm$ 3.8	59.3 $\pm$ 7.2	44 - 102 <sup>2</sup>
ATP content ( $\mu$ .moles)	) M e t h o d A	0.32 $\pm$ 0.03	2.57 <sup>3</sup>
adenylate content ( $\mu$ .moles)		2.46 $\pm$ 0.12	3.90
ATP/ADP		0.24 $\pm$ 0.02	2.95
ATP content ( $\mu$ .moles)	) M e t h o d B	0.76 $\pm$ 0.05	
adenylate content ( $\mu$ .moles)		2.98 $\pm$ 0.20	
ATP/ADP		0.73 $\pm$ 0.15	
Oxygen consumption ( $\mu$ .moles/min)	1.94 $\pm$ 0.09 <sup>4</sup>	4.73 $\pm$ 0.61	0.7 - 2.2 <sup>5</sup>
Glycogen content ( $\mu$ .moles)			
- FED	141.8 $\pm$ 17.2 <sup>6</sup>	150.4 $\pm$ 8.3	276 <sup>7</sup>
- 24h STARVED	7.1 $\pm$ 2.0 <sup>6</sup>	4.8 $\pm$ 2.2	

1 Personal communication, R.S. Campbell

2 Barnabei, et al, (1974)

3 Krebs, et al, (1974)

TABLE 3.10.

- 4 Bickerstaffe, et al, (1970)
- 5 Quistorff, et al, (1973)
- 6 Davison and Langslow, (1975)
- 7 Wagle, (1975)
- 8 Personal communication, S.A. Smith.

the low  $K^+$  in the intracellular fluid plus the small volume of the extracellular space ensures that this will not considerably affect the comparisons. The  $K^+$  content of isolated hepatocytes is 70-80% of whole liver when prepared by method A, and is insignificantly different in hepatocytes prepared by method B. A few authors have reported complete retention of  $K^+$  during rat hepatocyte isolation (Barnabei, et al, 1974) but most workers find that the isolation procedure lowers  $K^+$  content (Howard & Pesch, 1968; Berry & Friend, 1969; Krebs, et al, 1974). Slicing or mincing of liver causes  $K^+$  loss (Krebs, et al, 1974) and this may account for the losses observed to occur in chicken hepatocytes prepared by both methods A and B. Hepatocytes take up  $K^+$  during the first hour of incubation (Fig. 3.7) resulting in a  $K^+$  content of  $65.2 \pm 4.3 \mu\text{moles/g wet weight}$ , 85% of the whole liver value. The  $K^+$  uptake suggests that the hepatocytes continue to maintain ion gradients observed in whole liver and that the initial fall in  $K^+$  content is readily reversible.

The absence of a diaphragm in birds diminishes the ATP/ADP ratios in freeze-clamped livers unless artificially oxygenated (Table 3.5; Locke, et al, 1972; Söling, et al, 1973). Hepatocytes isolated by method A have ATP/ADP ratios similar to isolated rat hepatocytes but have an adenylate content of only 40% of that of whole liver (Table 3.10). Although Veneziale and Lohmar (1973) lost 75% of adenylates during isolation of rat hepatocytes, most workers have found adenylates to be retained totally (Quistorff, et al, 1973; Krebs, et al, 1974). The loss of adenylates may be due to their degradation by adenosine deaminase stimulated by high concentrations of ADP and AMP (Wood, et al, 1970) caused by the initial anoxia or due to the slicing of liver as suggested by Krebs, et al, (1974) (Table 3.5). The losses of ADP and

AMP that occurred during purification of hepatocytes by centrifugation (Table 3.2) may either be due to a washout from hepatocytes damaged during isolation (a similar pattern was observed for lactate dehydrogenase) or actual leakage from structurally intact hepatocytes.

The isolation of hepatocytes by the rapid procedures of method B resulted in total retention of adenylates (Table 3.10). Although both method A and B yielded cells with similar ATP contents, the ADP and AMP contents of those prepared by method B were much higher (Table 3.4). The rapidity of isolation of hepatocytes by method B may prevent leakage of ADP and AMP. As both slicing of liver (Table 3.5) and centrifugation can lower adenylate contents (Table 3.2), the reduction by 50% of centrifugation stages and the absence of slicing in isolation may cause greater adenylate retention. Whatever the cause(s), the use of method B to prepare hepatocytes results in preparations more similar to whole liver in terms of adenylate content than method A. The ATP/ADP ratio does not increase during incubation. This has also been found for the perfused chicken liver (Locke, et al, 1972). No satisfactory explanation for this low ATP content exists.

Endogenous oxygen consumption by the perfused chicken liver was significantly lower than that found for isolated chicken hepatocytes (Table 3.10). Permeation of gases may be increased by the increased surface area available for diffusion, the lack of possible intercellular controls and the unrestricted access to all cells in suspension. The average consumption of  $0.36 \pm 0.05 \mu \text{ moles } O_2/\text{hour}/10^6 \text{ hepatocytes}$  (Table 3.3) was greater than the value found for isolated chicken hepatocytes ( $0.21 \pm 0.08 \mu \text{ moles/h}/10^6 \text{ hepatocytes}$ ) by Badenoch-Jones and Buttery (1975). As the value is also greater than those obtained with isolated rat hepatocytes (Table 3.10), it is possible that

oxidative phosphorylation may be uncoupled. However as the stimulation of endogenous oxygen consumption by lactate (10 mM) was similar to isolated rat hepatocytes (Berry & Friend, 1969), any uncoupling present may be partial and not significantly different from that of other preparations.

In terms of dry weight, dimensions, protein and DNA contents (Table 3.3), chicken hepatocytes are smaller than rat hepatocytes (Berry & Friend, 1969; Capuzzi, et al, 1974). The physical characteristics of isolated chicken hepatocytes determined by Capuzzi, et al (1974) are considerably greater than those I report. However, the characteristics of rat hepatocytes isolated by those workers are also greater than those reported by other groups (Howard & Pesch, 1968; Seglen, 1973b; Krebs, et al, 1974).

#### Maintenance of hepatocytes during incubation.

Incubation of rat hepatocytes at 37°C resulted in an increase (4-15% per hour) in cells stained with trypan blue (East, et al, 1973). The values found for isolated chicken hepatocytes range from 10-15% and 20-28% with cells isolated from fed and 24 hour starved chickens respectively (Fig. 3.3). Given the possible variation in counting techniques, the rate of staining was similar for hepatocytes isolated by methods A and B (Fig. 3.4). However, the more rapid staining rate of hepatocytes from starved animals has not been reported previously for any species, although the observation that hepatocytes from starved rats formed membrane blisters more rapidly during incubation than those from fed rats (Wagle & Ingebretson, 1974) may be related to the alteration in membrane stability observed here.

Enzymes will be lost from hepatocytes with damaged cell membranes (Hoskins, et al, 1956). The extent of this damage was assessed from the leakage of lactate dehydrogenase and aspartate aminotransferase (cytoplasmic) and glutamate dehydrogenase (mitochondrial) from the cells. The close correlation found between leakage of cytoplasmic enzymes and trypan blue entry suggests (Fig. 3.6) that the cells cytoplasmic contents equilibrate with the surrounding medium. The rate of glutamate dehydrogenase leakage was slower than the other two enzymes (Fig. 3.5) and its correlation with staining was not as good (Fig. 3.6). This suggests that while cytoplasmic enzymes leaked out of cells, a percentage of glutamate dehydrogenase was retained either due to intact mitochondria or to bound enzymes. However following trypan blue entry a large proportion of mitochondrial enzyme is released into the surrounding medium, and the stained cells must have greatly impaired metabolic functions.

As the number of hepatocytes excluding trypan blue decreased there were simultaneous decreases in several metabolic functions (Fig. 3.7). The decrease in endogenous oxygen consumption was considerably more rapid in hepatocytes from 24 hour starved chickens than fed (Table 3.6) presumably reflecting the more rapid degeneration observed with staining. The maintenance of total adenylate content, while ATP content decreased, suggests that cell degradation leads to the conversion of ATP to ADP and AMP. Thus the retention of ADP and AMP in the medium would explain the decrease in the ATP/ADP ratio observed during incubation (Table 3.7).

Glucose was released into the medium and the glycogen content of the hepatocyte fell (Fig. 3.8, and see Chapter 4 and 5). More glycogen was lost than glucose recovered, presumably since some glucose is oxidised. The glycogen content of hepatocytes from starved chickens

did not change during incubation. They presumably utilised some other energy source.

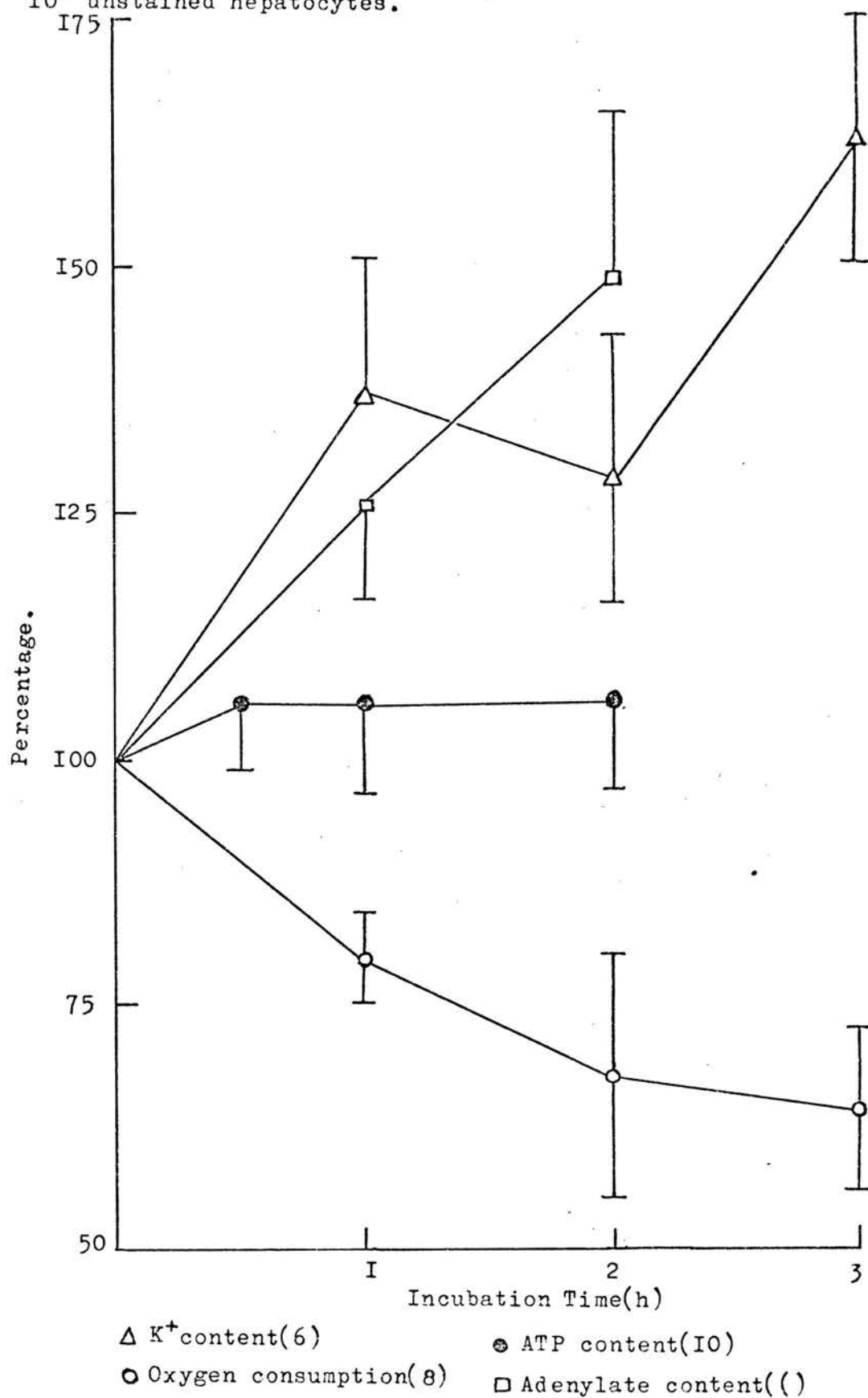
#### Metabolic integrity of preparations

Hepatocytes isolated by method B are metabolically similar in many ways to whole liver. The biosynthesis of glucose indicates the integrity of isolated liver preparations (Krebs, et al, 1974), especially from lactate as it involves both mitochondrial and cytosolic compartments (Ross, et al, 1967). If permeability barriers are retained during isolation of liver preparations, glucose synthesis from di- and tricarboxylic acids is negligible (Ross, et al, 1967; Garrison & Haynes, 1973). The ability of isolated chicken liver cells to synthesize glucose and their sensitivity to glucagon (see Chapters 4, 5 and 6) together with their metabolic similarity to whole liver, suggests that these preparations offer a valuable system for the study of hepatic metabolism and its control.

The preparations are limited to some extent by the degenerative changes occurring during incubation. These alterations are unaffected by the presence of hyaluronidase during the isolation of hepatocytes or by substrate availability during incubation. The treatment of isolated rat hepatocytes with digitonin resulted in preparations totally permeable to trypan blue (Mapes & Harris, 1975). Although only a simulation of the natural processes which result in staining, the study of digitonin-treated cells indicated that trypan blue stained hepatocytes are metabolically inactive. During incubation the uptake of trypan blue resulted in a loss of enzymes, ATP and  $K^+$  content and a fall in endogenous oxygen consumption. The hepatocytes are then probably metabolically inactive. The time course of these changes is unknown

FIG. 3.II Effect of incubation on metabolic characteristics.

Results of Fig. 3.7 recalculated in terms of units/  
 $10^6$  unstained hepatocytes.





and the process may be gradual with hepatocytes going through distinct stages of degeneration. Homogenates of pigeon liver synthesised glucose from lactate but chicken liver homogenates did not (Krebs, et al, 1964). Even if certain stages of hepatocyte degeneration resemble homogenate conditions, they are unlikely to be biosynthetically active without the addition of cofactors necessary in homogenates.

The degenerative changes highlight the difficulties in the expression of results. Expression in terms of dry weight or protein content gives no indication of the quality of preparations prior to or during incubations. Light microscopic examination permits a rapid and accurate method of assessing preparation quality since it correlates with many metabolic characteristics. It shows that suspensions contain an altering proportion of stained and unstained hepatocytes during incubation. "Half-lives" calculated from staining rates were 5.25 hours for fed chicken hepatocytes and 2.5 hours for 24 hour starved.

#### Metabolic activity of unstained hepatocytes

Metabolic activities in terms of units per  $10^6$  unstained hepatocytes were calculated from the results of Fig. 3.7 (Fig. 3.11). Using unstained hepatocytes as a unit reference, ATP content remained constant during incubations. While the increase in total adenylate content could be caused by adenylate synthesis by unstained hepatocytes it is probably due to retention of ADP and AMP released from stained hepatocytes. The increased content of intracellular  $K^+$  during incubation indicated that stained hepatocytes either did not release all their  $K^+$  (for example mitochondrially located  $K^+$ ) or that unstained hepatocytes were initially deficient in  $K^+$ .

TABLE 3.11. Effect of incubation on oxygen consumption.

Hepatocytes isolated with 0.25mg collagenase/ml with or without 1.0mg hyaluronidase/ml. Means  $\pm$  SEM's calculated as units per  $10^6$  unstained hepatocytes, and expressed as percentage of the initial value.

<u>CHICKEN</u>	<u>HYALURONIDASE</u>	<u>INCUBATION TIME(h)</u>		
		<u>1</u>	<u>2</u>	<u>3</u>
FED	- (8)	79.0 $\pm$ 5.5	66.6 $\pm$ 12.2	64.6 $\pm$ 10.2
	+ (8)	83.6 $\pm$ 8.8	75.5 $\pm$ 9.4	62.8 $\pm$ 16.6
24h.				
STARVED	- (4)	105.9 $\pm$ 5.4	-	102.9 $\pm$ 7.7

Hepatocytes were prepared by method A and the number of experiments are given in parentheses.

Endogenous oxygen consumption fell for fed chicken hepatocytes when calculated in terms of unstained hepatocytes, but not for 24 hour starved chicken hepatocytes (Table 3.11). The reason for this difference is unknown.

The correlation between trypan blue staining and metabolically altered hepatocytes, indicates that the number of unstained hepatocytes provides the most satisfactory unit for the expression of results. Although the reason for hepatocyte instability is unknown it may be caused by damage to cell surfaces by impurities in collagenase, loss of intercellular contacts, the initial anoxia or general mechanical stress during isolation.

#### SUMMARY

A number of methods have been used to assess the metabolic integrity of isolated chicken hepatocytes. Many of the methods were optimised to provide sensitive indicators of viability. To accurately reflect the quality of the isolated cell preparations, their characteristics were compared to those of whole chicken liver. Immediately after isolation, chicken hepatocytes were similar to whole liver. The major difference was caused by the effects of the isolation procedures on the hepatocyte adenylate content and ATP/ADP ratio. In this, however, the method used for preparation was crucial. Method B resulted in normal adenylate contents compared to whole liver but with low ATP/ADP ratios due to anoxia during isolation. Apart from this factor, method B resulted in hepatocytes almost identical to whole liver.

During incubation hepatocytes undergo degenerative changes that can be correlated with loss of metabolic activity and permeation of trypan

blue. The microscopic examination of preparations for trypan blue uptake provides a rapid and sensitive assessment of preparation quality. The metabolic changes associated with trypan blue uptake suggest that unstained hepatocytes provide the preferable unit of reference for results of metabolic studies. With this condition, isolated chicken hepatocytes should prove useful for short and long-term (4 hours) metabolic investigations, and provide results that may be confidently extrapolated to the whole chicken liver.

Except when specifically stated in the text, tables or figures, the results described throughout the rest of this thesis were obtained with cells prepared by method B.

## CHAPTER 4

### GLUCOSE PRODUCTION BY HEPATOCYTES FROM FED CHICKENS

### INTRODUCTION

The pathway of gluconeogenesis in isolated chicken hepatocytes and its control was investigated by measuring glucose production from substrates known to enter the gluconeogenic pathway at different points (Fig. 4.1). Endogenous and substrate-stimulated glucose production was measured with fed and starved chicken hepatocytes. The substrate effects were correlated with reported substrate effects on plasma glucose concentrations in vivo. This chapter describes glucose production by fed chicken hepatocytes, while the following chapter details glucose production by hepatocytes from starved chickens. Chapter 5 also contains a general discussion of the gluconeogenic capacity of isolated chicken hepatocytes.

### METHODS

Substrates and other additions were made with Hamilton microsyringes (10  $\mu$ l aliquots) to give the desired final concentration. Experiments on substrate effects determined the maximum rates of glucose production from each precursor as well as the production over differing time intervals. The choice of a single incubation time can be critical for accurate interpretation of results, and was highlighted by the 20 minutes lag in gluconeogenesis from lactate by isolated rat hepatocytes. (Johnson, et al, 1972; Garrison & Haynes, 1973; Cornell, et al, 1974).

Endogenous glucose production is expressed as  $\mu$ g glucose produced per  $10^6$  unstained hepatocytes. Substrate effects are expressed as increases over the endogenous production to overcome the day to day differences in basal glucose production. Glycogen content is given as

FIG. 4.I Points of entry of glucose precursors into the gluconeogenic pathways of rat and chicken liver.

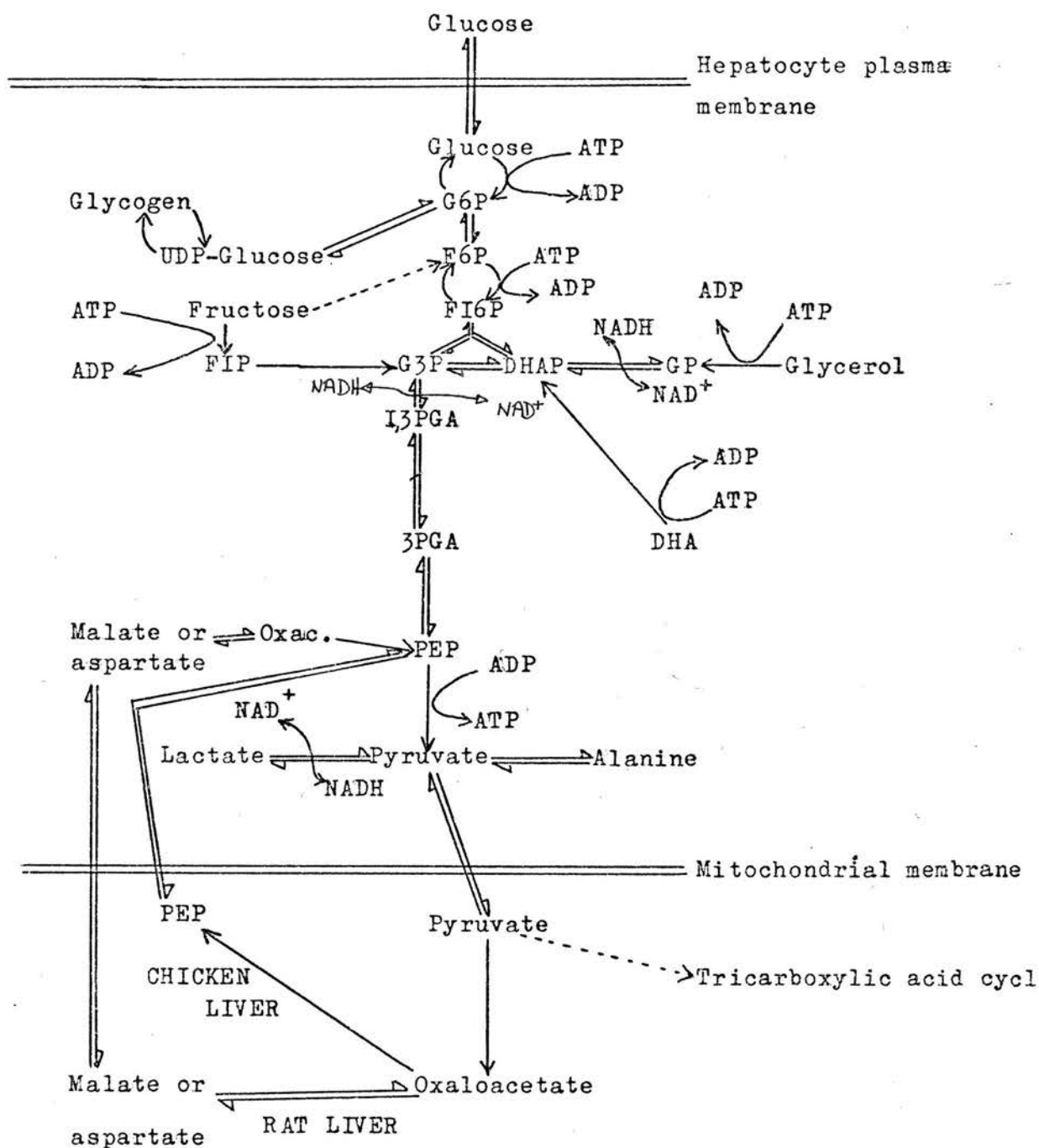


FIG. 4.2 Time course of glucose production and glycogen loss.

Glucose(●) and glycogen( $\Delta$ ) values are means of 17 experiments  $\pm$  SEM's.

Hepatocytes were prepared from fed chickens.

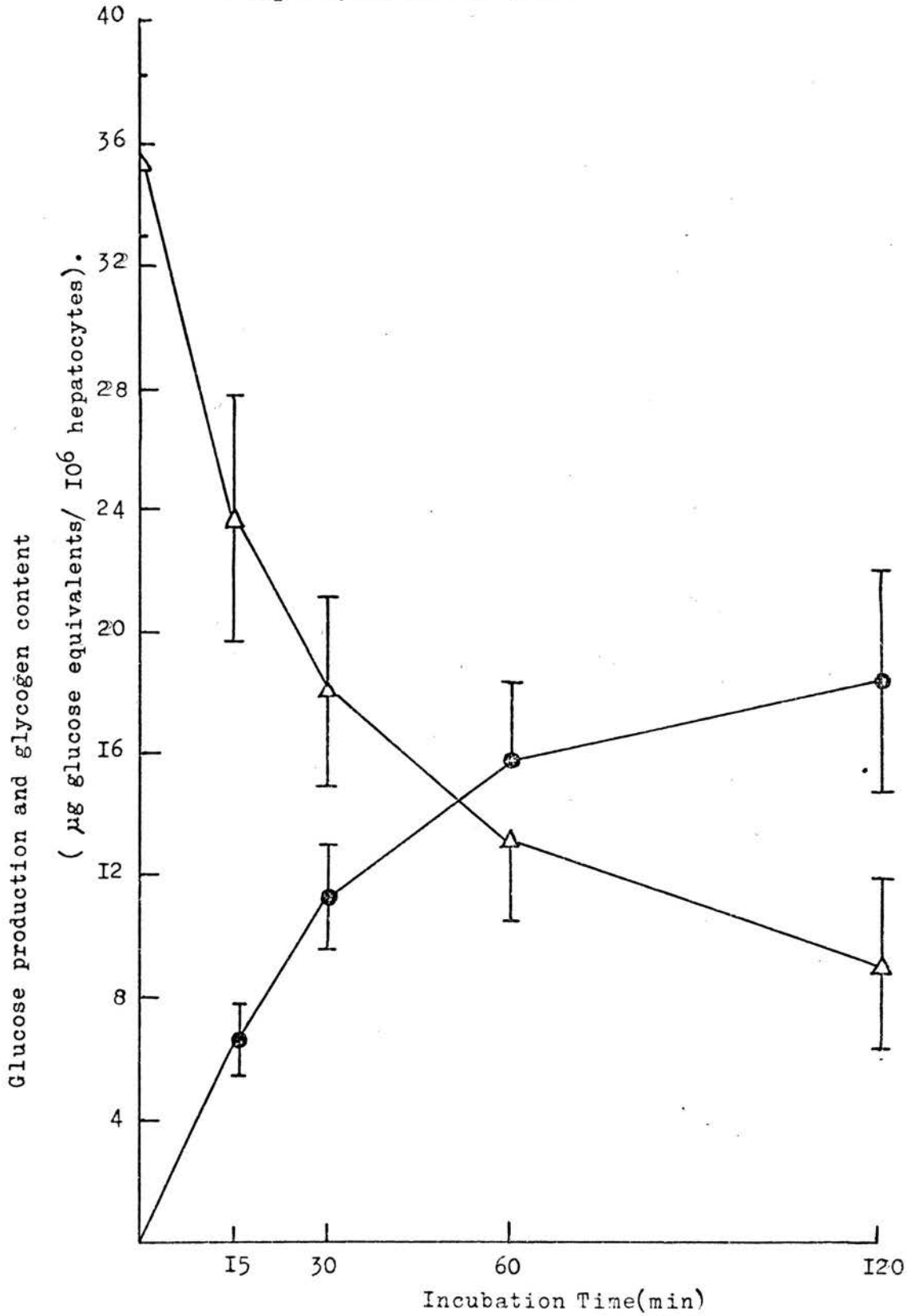




FIG. 4.3 Correlation between initial glycogen content and  
the rate of glucose release of fed chicken hepatocytes

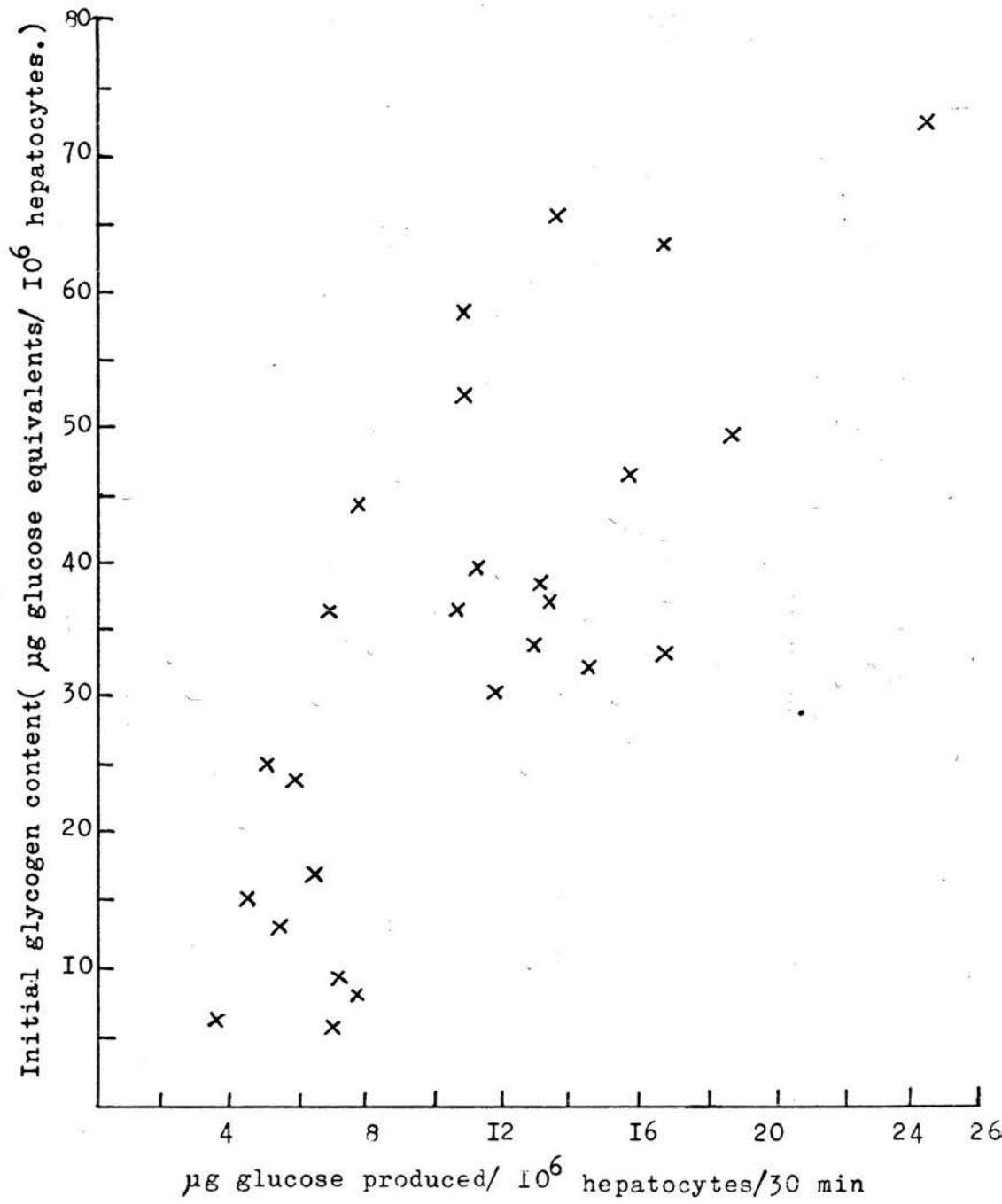
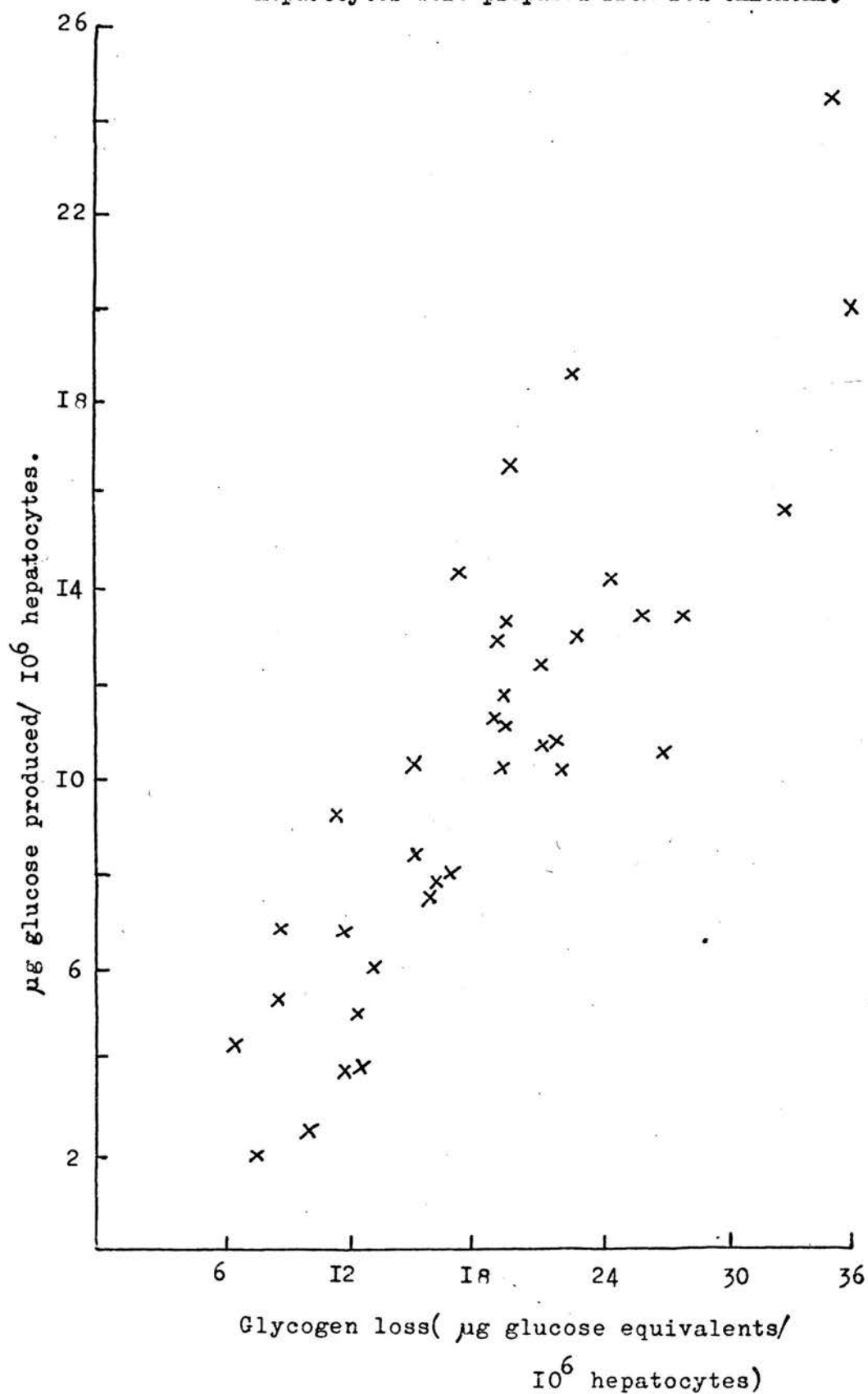


FIG.4.4 Correlation between glucose production and glycogen

loss. Hepatocytes were prepared from fed chickens.



$\mu\text{g}$  glucose equivalents per  $10^6$  unstained hepatocytes and substrate effects as alterations from basal. These conventions are retained in Chapters 5 and 6 also.

Hepatocytes from fed chickens were found to contain more glycogen if isolated in the afternoon. Diurnal rhythms of liver glycogen content occur in birds (Hazelwood, 1972) and mice (Krebs, *et al*, 1966). Thus experiments with fed chicken hepatocytes were always started around 13.00 hours to ensure a high glycogen content.

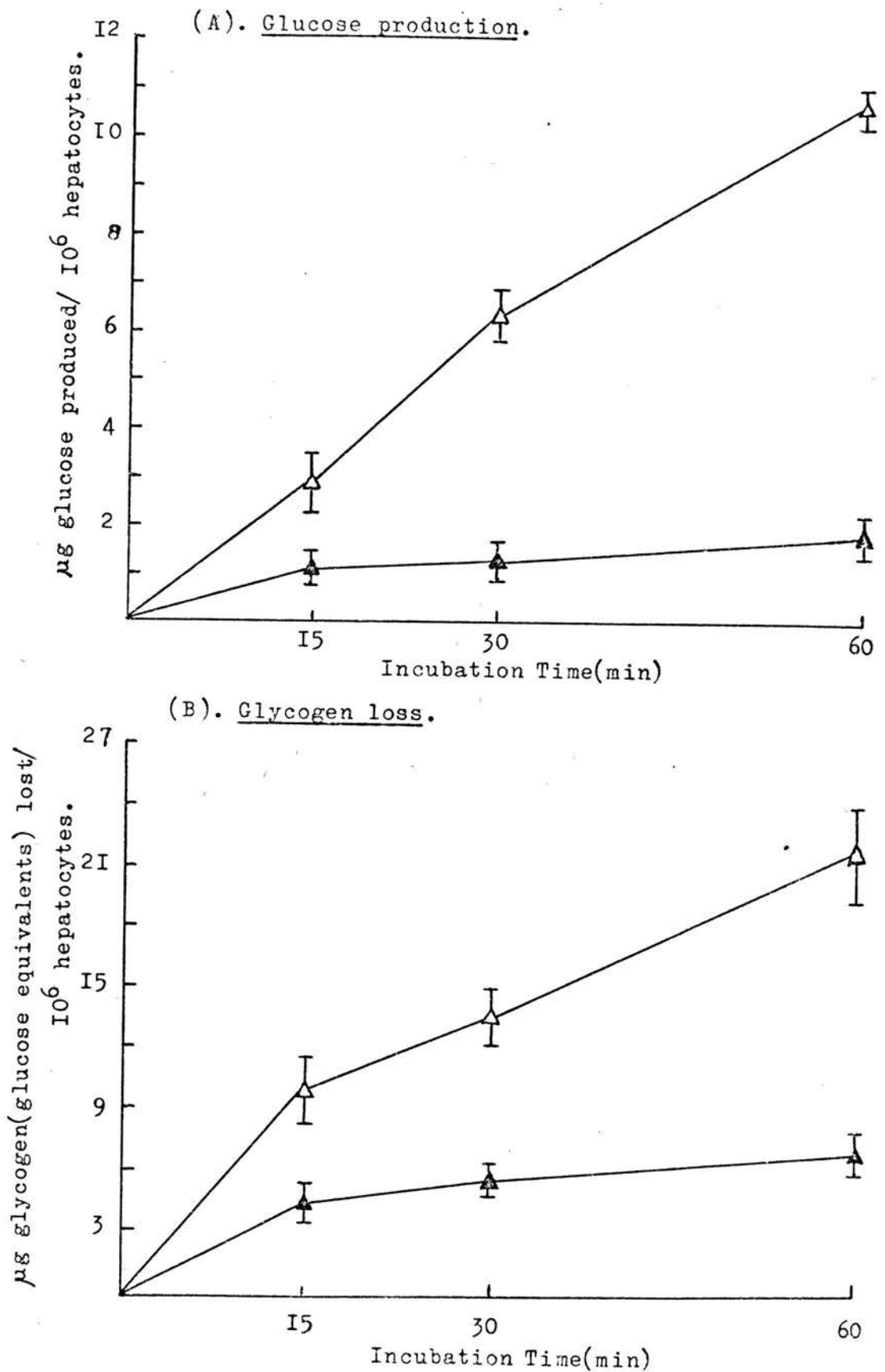
## RESULTS

Hepatocytes from fed chickens lost glycogen and the glucose content of the medium increased during incubation (Fig. 4.2). The rate of glucose production depended on the initial glycogen content (Fig. 4.3). The changes in glucose and glycogen contents were rapid over the first 30 minutes and then progressively slowed (Fig. 4.2), and the glucose production correlated with the glycogen loss (Fig. 4.4). More glycogen disappeared than was produced as glucose. This "missing" glucose was probably metabolised by the cells. The preparation and incubation of hepatocytes in modified KRB with high  $\text{K}^+$ /low  $\text{Na}^+$  dramatically decreased glycogenolysis (Fig. 4.5). Details of this buffer are given on page 22.

Throughout incubation pyruvate, lactate and dihydroxyacetone (DHA) increased glucose production (Fig. 4.6). The rate of production often decreased after the first 15 minutes and resembled the decrease in endogenous glucose production (Fig. 4.2). Glycerol did not alter glucose production whereas alanine caused an increase midway between those of glycerol and lactate. There was a lag phase (15 min) in glucose production with fructose, after which a rapid and constant rate

FIG. 4.5 Effect of high- $K^+$  KRB on glycogenolysis.

Values are means  $\pm$  SEM for 3 experiments. Hepatocytes incubated in normal(low  $K^+$ ) KRB( $\Delta$ ) or high- $K^+$  KRB( $\blacktriangle$ ).

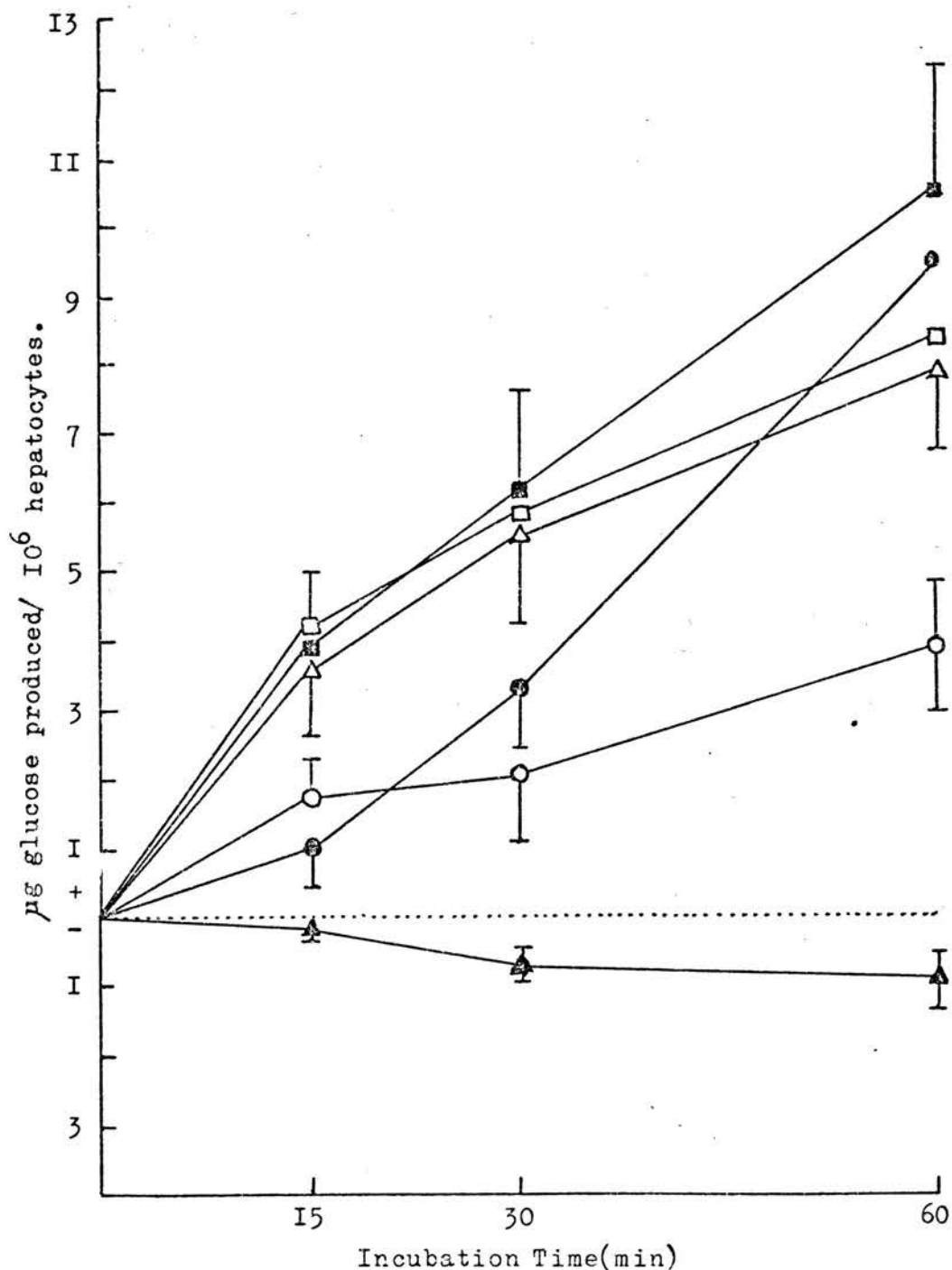


Hepatocytes from fed chickens were both prepared and incubated in the relevant KRB.

FIG. 4.6 Time course of substrate effects on glucose production.

Substrates added to give a final concentration of 10mM and results expressed as differences from the control value at each time. Values are means  $\pm$  SEM's with number of experiments in parentheses.

Hepatocytes were prepared from fed chickens.



- |               |                       |
|---------------|-----------------------|
| △ Lactate(3)  | ▲ Glycerol(6)         |
| □ Pyruvate(4) | ■ Dihydroxyacetone(5) |
| ○ Alanine(3)  | ● Fructose(8)         |

FIG. 4.7 Effect of varied substrate concentrations on glucose production of fed chicken hepatocytes.

Hepatocytes incubated for 30 min and results expressed as difference from basal value. Values are means  $\pm$  SEM's (Numbers of observations in parentheses).

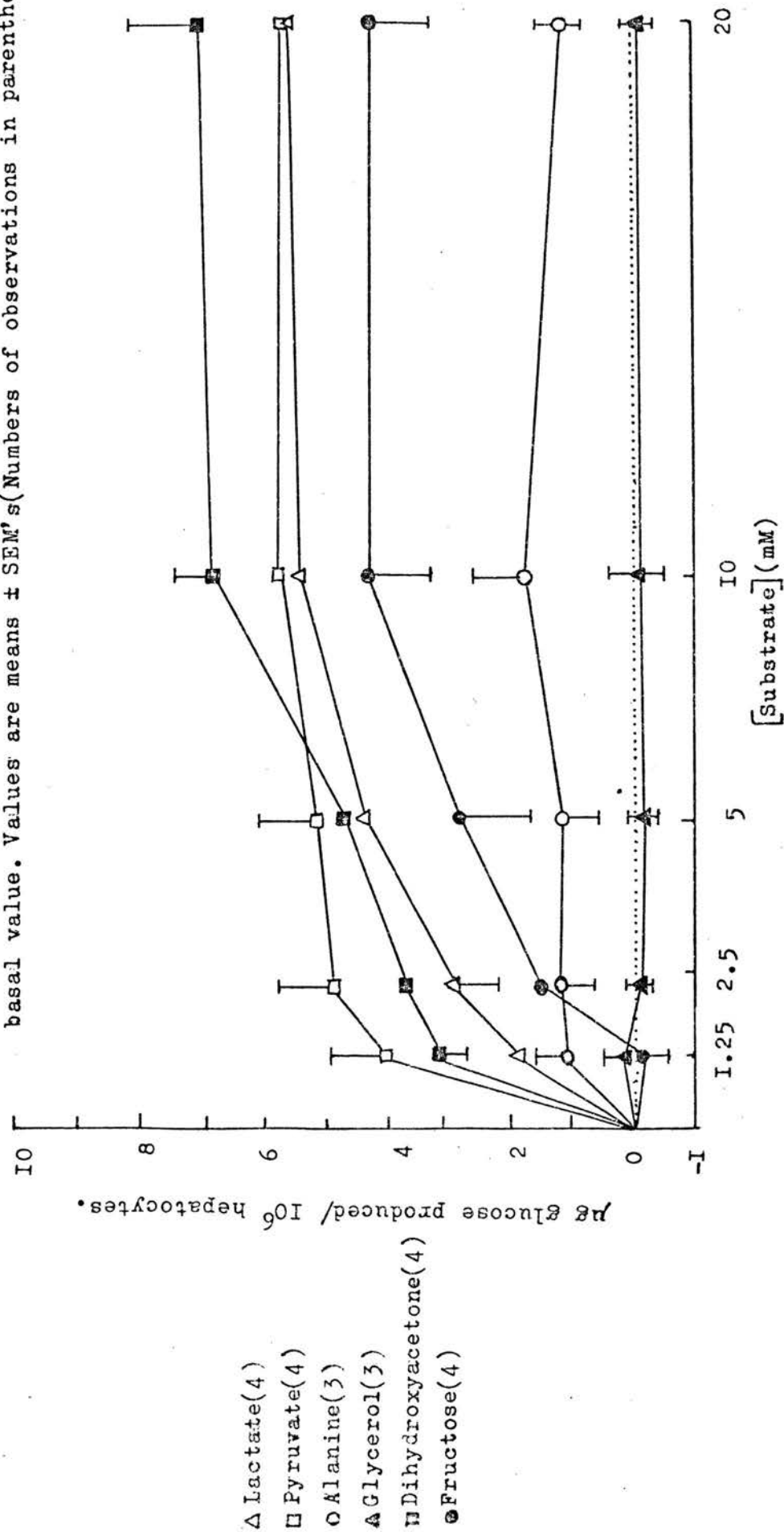


TABLE 4.1. Effects of substrate addition on hepatocyte glycogen content.

Substrates (10mM) added to suspensions. After 30 minutes incubation, glycogen contents were measured and results expressed as difference from control glycogen content. Values are means  $\pm$  SEM's ( $\mu$ g glucose- equivalents/ $10^6$  hepatocytes) and number of experiments are given in parentheses. Hepatocytes were prepared from fed chickens.

<u>SUBSTRATE</u>		<u>DIFFERENCE FROM CONTROL</u>
Lactate	(3)	-0.71 $\pm$ 0.61
Pyruvate	(4)	-1.39 $\pm$ 0.60
Alanine	(3)	-3.95 $\pm$ 1.37
Glycerol	(5)	+1.49 $\pm$ 0.16
Dihydroxyacetone	(5)	+1.19 $\pm$ 0.60
Fructose	(8)	+3.81 $\pm$ 0.84

TABLE 4.2. Effects of fructose on glycogen content.

Results expressed as in Table 4.1.

A. Time course of fructose effect.

Values are mean difference from control glycogen ( $\mu\text{g}$  glucose-equivalents/ $10^6$  hepatocytes)  $\pm$  SEM for 4 experiments with 10mM fructose.

<u>Incubation Time (min)</u>	<u>Fructose effect on glycogen</u>
15	+0.88 $\pm$ 1.13
30	+2.91 $\pm$ 2.07
60	+6.07 $\pm$ 2.27
120	+12.61 $\pm$ 3.59

B. Effect of varied fructose concentrations.

Hepatocytes incubated in the presence or absence of fructose for 30 minutes and the results expressed as above.

<u>Fructose concentration (mM)</u>	<u>Fructose effect on glycogen</u>
1.25	+0.65 $\pm$ 0.22
2.5	+1.11 $\pm$ 0.51
5	+4.67 $\pm$ 0.81
10	+5.02 $\pm$ 0.87
20	+4.08 $\pm$ 1.23

Hepatocytes were prepared from fed chickens. Values are means  $\pm$  SEM from 3 experiments.



TABLE 4.3. Effect of incubation buffer potassium and glucose content on hepatocyte glycogen loss.

Hepatocytes were incubated in normal KRB or in modified KRB (high K<sup>+</sup>/low Na<sup>+</sup>) for 2 hours and the effects of exogenous glucose on glycogen loss measured. Values are means for 3 experiments  $\pm$  SEM's. (-, loss; + gain of glycogen)

Hepatocytes were prepared from fed chickens.

<u><math>\mu</math>g glycogen(glucose-equivalents) lost during</u>		
<u>2h incubation/<math>10^6</math> hepatocytes.</u>		
<u>Glucose (mM)</u>	<u>Normal KRB</u>	<u>Modified KRB</u>
0	-15.90 $\pm$ 0.51	-10.12 $\pm$ 0.98
10	-14.50 $\pm$ 0.84	- 2.36 $\pm$ 0.66
20	-14.81 $\pm$ 0.62	- 1.46 $\pm$ 1.71
40	-11.98 $\pm$ 0.47	- 0.32 $\pm$ 0.92
60	-11.08 $\pm$ 0.67	+ 2.84 $\pm$ 2.31

was attained (Fig. 4.6). With all substrates maximal effects were observed at 10mM (Fig. 4.7).

The effects of substrates on the glycogen content of hepatocytes after 30 minutes incubation with these substrates is shown in Table 4.1. Only fructose significantly prevented glycogen degradation. This effect was observed throughout a 2 hour incubation period, after a 15 minute lag period, and was dependent on the fructose concentration (Table 4.2). Considering both glucose production and glycogen retention, the effectiveness of the substrates is as follows:

fructose > DHA > lactate = pyruvate > alanine = glycerol

Incubation of hepatocytes with glucose (10-60mM) for 2 hours and incubation was ineffective in increasing glycogen (Table.4.3). Preparation of hepatocytes in modified KRB with high  $K^+$ /low  $Na^+$  resulted in much greater retention of glycogen in the presence of glucose but there was no evidence for significant net synthesis.

## DISCUSSION

Although perfused liver and liver slices from fed chickens produce glucose, the investigations were of a qualitative rather than a quantitative nature (Bickerstaffe, et al, 1970; Bannister, et al, 1975). The studies of glycogenolysis in isolated rat liver preparations offer a more useful comparison to the data presented here.

The effects of substrates on glucose production are complicated by the high rates of glycogenolysis in hepatocytes from fed chickens. Apart from small effects being difficult to distinguish from endogenous effects (a measurement problem), increased glucose production may be due not only to glucose synthesis but also to added substrates sparing

glucose oxidation. They could also possibly have direct effects on, for example, glycogen phosphorylase. However, substrate addition never significantly increased the rate of glycogen degradation (Table 4.1) and as the time course for increased glucose production parallels that of endogenous glucose production, the glucose sparing effect is likely to be most important.

Fructose performs differently from other substrates. The lag in glucose production has also been found to occur in the perfused rat liver (Sestoft, 1974). In both chicken and rat liver fructokinase (E.C. 2.7.1.3) phosphorylates most of the fructose metabolised (Hazelwood, 1972). In the perfused rat liver, fructose (10mM) infusion resulted in a 70% fall in ATP content and an accumulation of fructose-1-phosphate (Woods, et al, 1970; Sestoft, 1974). As fructose-1-phosphate inhibits phosphorylase (van den Berghe, et al, 1973), glycogenolysis would be decreased and this effect of fructose has been observed with the perfused liver and isolated hepatocytes from rat (Woods & Krebs, 1972; Howard & Widder, 1976). The extrapolation of these effects to chicken hepatocytes may not be entirely valid, as fructose decreased the ATP content of isolated chicken hepatocytes by only 10-15% (Table 3.9B). However as the initial ATP content of isolated chicken hepatocytes is much lower than that of rat liver, the effect of fructose on ATP content might be less dramatic. Thus it is possible that an inhibition of glycogen phosphorylase by accumulated fructose-1-phosphate may occur in isolated chicken hepatocytes.

Hepatocytes isolated from fed rats undergo rapid glycogenolysis and lose 50% of their glycogen within one hour of incubation (Johnson, et al, 1972; Garrison & Haynes, 1973). Glycogen loss was not exactly

balanced by glucose production in these preparations and although the rate of glycolysis is low in isolated rat liver preparations (Schimassek, et al, 1974; Seglen, 1974), the unaccounted glucose is presumably oxidised. Glucose production from glycogen breakdown will be determined between the rates of conversion of glucose-6-phosphate to lactate (which is controlled at the level of phosphofructokinase) and hydrolysis by glucose-6-phosphatase. Normally the activity of phosphofructokinase will be more rate limiting for glucose-6-phosphate metabolism.

At some stage in the preparation of rat hepatocytes the tissue becomes anoxic (Seglen, 1973C) and isolated chicken hepatocytes were anoxic (low ATP/ADP ratios - Table 3.4). This may increase the rate of glycolysis of the preparation (Pasteur effect). The activation of glycogen phosphorylase (70% active form) and inactivation of glycogen synthetase (10% active form) as a result of rat hepatocyte isolation procedures may serve to increase glucose-6-phosphate supply for glycolysis immediately after isolation (Hue, et al, 1975). During incubation phosphorylase activity decreased and synthetase I activity increased. The changes in activity were more rapid when wells were incubated with modified KRB (high  $K^+$ /low  $Na^+$ ) and the effects observed with modified KRB on chicken hepatocytes (Fig. 4.5) may also be related to alterations in phosphorylase and glycogen synthetase activities during incubation.

The extracellular fluid glucose concentration is a major factor controlling glycogen metabolism of rat liver (Hers, 1976), and increased the rate of inactivation of phosphorylase in isolated rat hepatocytes (Hue, et al, 1975). In isolated chicken hepatocytes, glucose (10-60mM) had smaller effects on glycogen content than fructose. In the presence of

modified KRB (high  $K^+$ /low  $Na^+$ ), glucose prevented glycogen degradation but it is unlikely that any net synthesis of glycogen occurred (Table 4.3). Glycogen synthetase is inactive in rat hepatocytes until less than 10% of the phosphorylase remains active (Hue, et al, 1975). The lack of glycogen synthesis by chicken hepatocytes may reflect the retention of high phosphorylase activity.

#### SUMMARY

The glycogen content of hepatocytes isolated from fed chickens fell rapidly during incubation. A proportion of the glucose units released from glycogen equilibrated with glucose in the extracellular medium. The rest was presumably metabolised by the cells. The rapid glycogenolysis was probably caused by anoxia during cell preparation which resulted in low ATP/ADP ratios. This is likely to accelerate the glycolytic flux and activate glycogen breakdown to provide substrates for metabolism. Added substrates increased the flux of glucose into the extracellular pool possibly by a substrate-sparing effect on glucose-units released from glycogen. Glycogen synthesis did not occur and this may be a consequence of the rapid rate of glycogen breakdown.

## CHAPTER 5

### GLUCOSE PRODUCTION BY HEPATOCYTES

#### FROM STARVED CHICKENS

### INTRODUCTION

Hepatocytes from fed chickens are unsuitable for studying substrate effects on glucose production without using radioactively labelled precursors. The low glycogen content of hepatocytes from starved chickens (Table 3.3) allows gluconeogenesis from added precursors to be measured with more precision than possible with fed chicken hepatocytes.

The ratios of  $\text{NADH/NAD}^+$  and  $\text{ATP/ADP.AMP}$  are important for determining the rates of many enzyme reactions, and have been postulated to play a major role in the overall control of metabolic fluxes (Atkinson, 1966). Ethanol increases the cytoplasmic  $\text{NADH/NAD}^+$  ratio (Williamson, et al, 1969b) whereas the ratio is decreased by acetaldehyde (Krebs, et al, 1969). Adenosine increases hepatic adenylate content and this effect has been observed with isolated rat hepatocytes (Lund, et al, 1975). This chapter describes the investigation of gluconeogenesis from a wide range of possible precursors and their interactions with one another. The effects of altered  $\text{NADH/NAD}^+$  ratios and increased adenylate contents on gluconeogenesis from these substrates was studied. The final section of the chapter contains a discussion of specific aspects of gluconeogenesis relevant to glucose production from substrates by both fed and starved chicken hepatocytes.

### METHODS

Substrate additions and incubations were carried out as described for fed chicken hepatocytes. Ethanol and acetaldehyde were added immediately before incubation began. The gluconeogenic ability of liver slices prepared from 24 hour starved chickens was determined

FIG. 5.I Time course of glucose production from 24h starved chickens.

Substrates added to give a final concentration of 10mM, and results expressed after subtraction of basal.

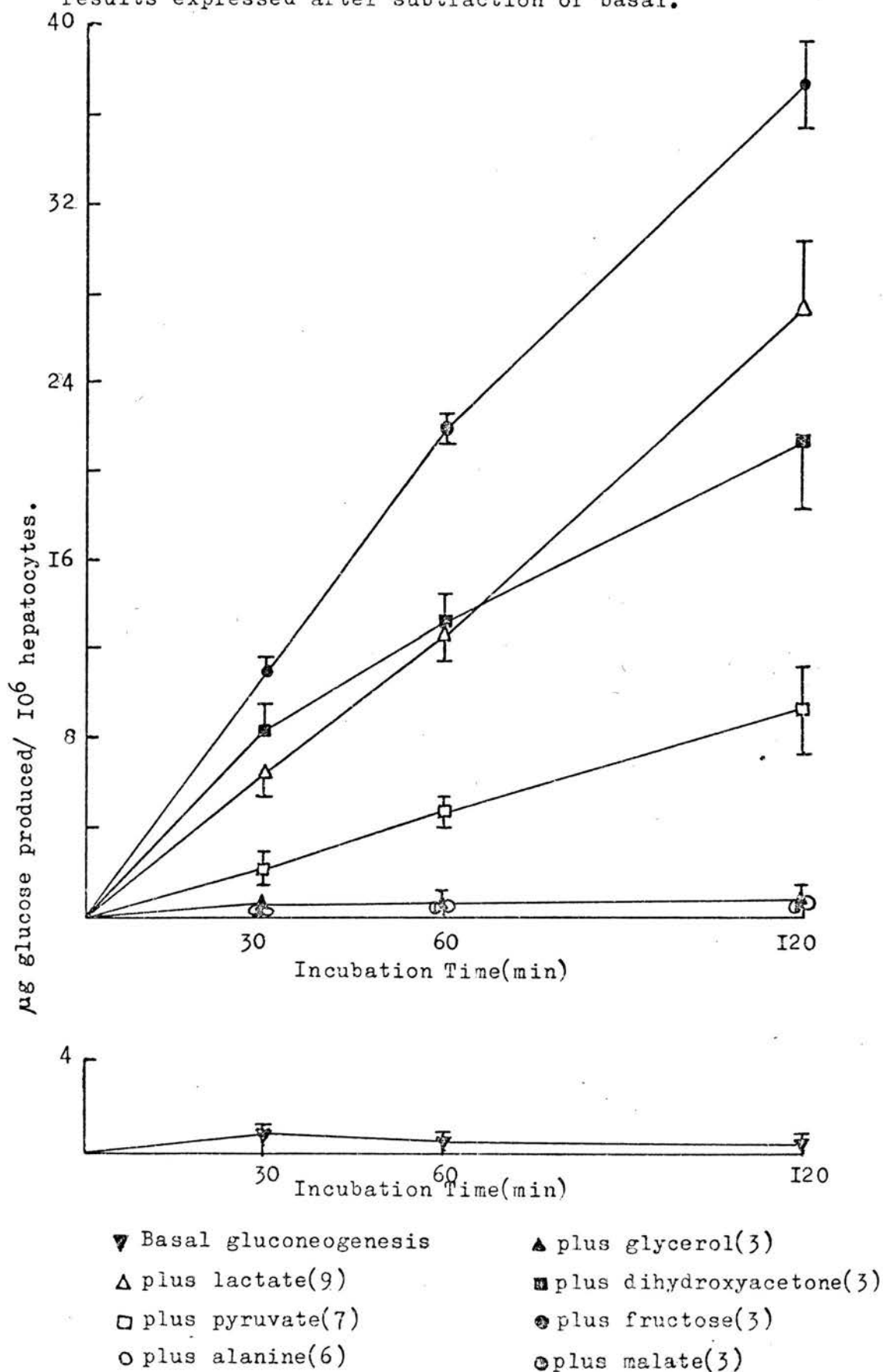
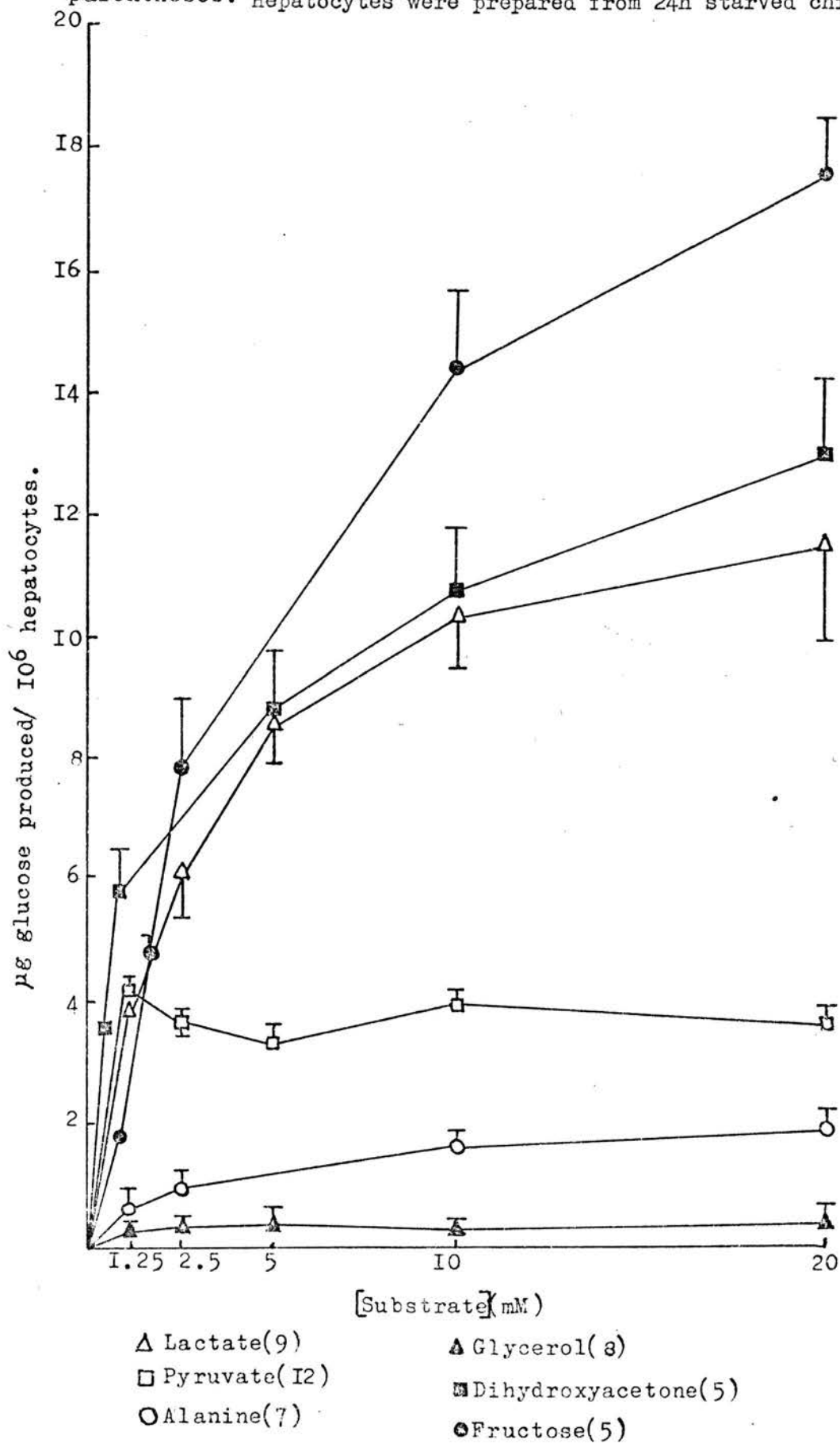




FIG. 5.2 Effect of substrate concentration on gluconeogenesis.

Values are mean glucose production during a 30 min incubation  $\pm$  SEM. Number of experiments given in parentheses. Hepatocytes were prepared from 24h starved chickens.



with slices (50-100 mg) prepared as described in Chapter 2. After oxygenation the slices were incubated in 2 ml KRB with 2% BSA for 60 min at 40°C and the reaction terminated with 2 ml 10% trichloroacetic acid. The samples were centrifuged (5 min at 2750 g), the supernatant extracted three times with ether and aliquots of the supernatant were assayed for glucose.

Glycerokinase activity was assayed in 20% homogenates of chicken liver and kidney by the radiochemical method of Newsholme, *et al* (1967). Extraction and assay buffers were those given by Robinson and Newsholme, (1969).

## RESULTS

### Substrate Effects

Almost no glucose was produced endogenously by hepatocytes from 24 hour starved chickens and no changes were detected in their glycogen content (Fig. 5.1). When alanine, DHA, glycerol, lactate, malate and pyruvate were added glucose was produced at constant rates over two hours (Fig. 5.1). Gluconeogenesis from fructose decreased between the first and second hours of incubation. Unlike the situation with fed chicken hepatocytes, there was no lag in glucose production from fructose.

Apart from fructose and DHA, all substrates had maximal effects of glucose production at 10 mM (Fig. 5.2). The gluconeogenic activity of the precursors was similar to that of total glucose production by fed chicken hepatocytes with fructose>DHA>lactate>>alanine>glycerol. However, unlike the fed chicken hepatocytes, pyruvate was only 30% as active as lactate, and produced maximal amounts of glucose at low concentrations (Table 5.1A).

FIG. 5.3 Time course of glucose production from 48h starved chickens.

Substrates added to give final concentration of 10mM.

- ▼ Basal gluconeogenesis(4)      □ plus pyruvate(3)  
 Δ plus lactate(4)      ▲ plus glycerol(3)

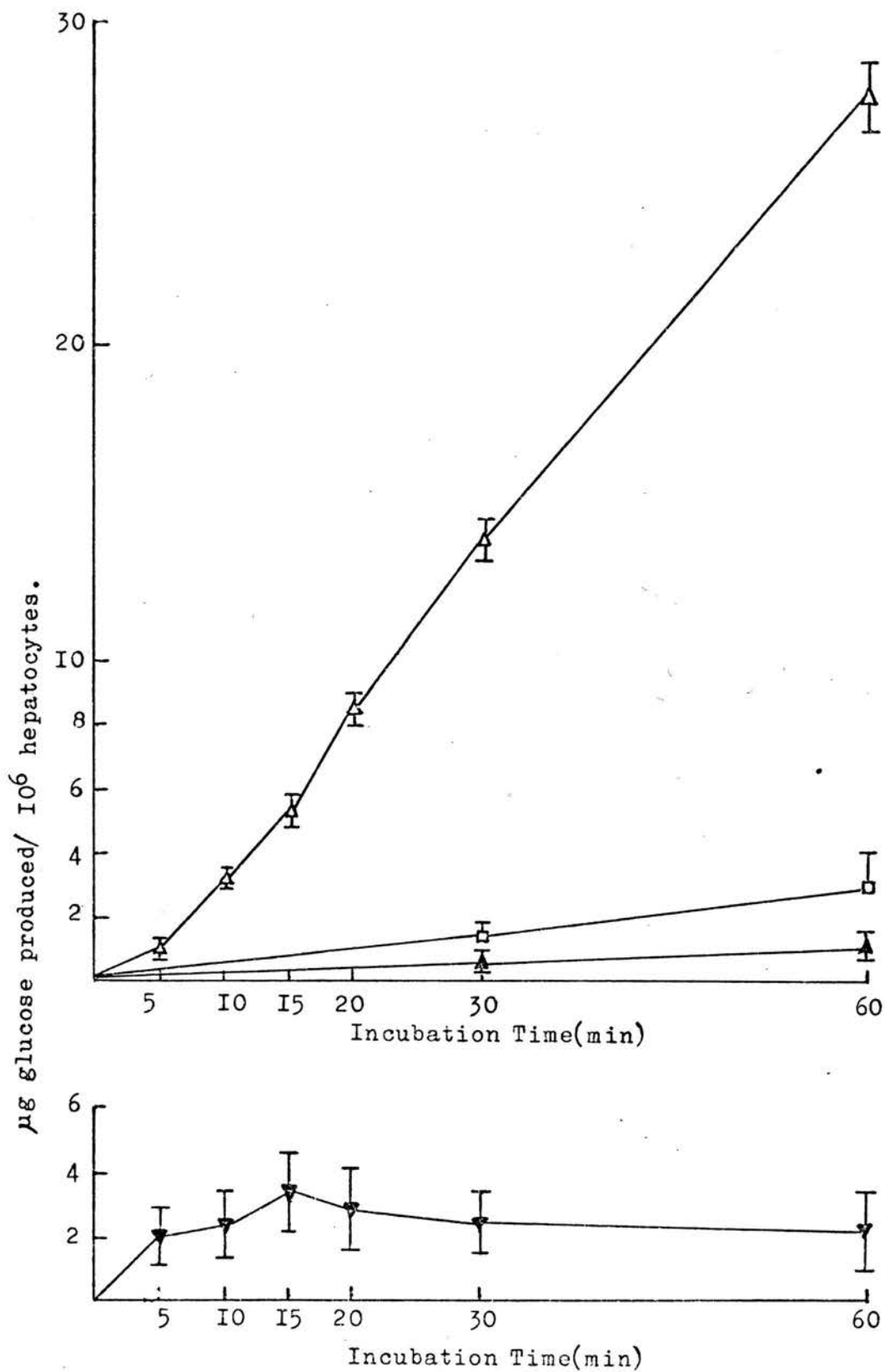


TABLE 5.1. Effects of a range of substrate concentrations on glucose production by hepatocytes from 24h starved chickens.

Hepatocytes were incubated for 30 minutes in the presence or absence of substrates. Results are expressed as the difference from the control glucose production ( $\mu\text{g}$  glucose produced/ $10^6$  hepatocytes) and are means  $\pm$  SEM's. The number of experiments are given in parentheses.

A. Varied pyruvate and hydroxypyruvate

	CONCENTRATION (mM)									
<u>Substrate</u>	<u>0.15</u>	<u>0.25</u>	<u>0.31</u>	<u>0.625</u>	<u>1.25</u>	<u>2.5</u>	<u>5</u>	<u>10</u>	<u>20</u>	
Pyruvate	1.85	2.28	3.77	4.68±0.31	4.13	3.62	3.35	3.92	3.61	
(12)	±0.16	±0.19	±0.23		±0.22	±0.21	±0.28	±0.27	±0.26	
Hydroxypyruvate	0.09±0.15		1.46±	1.89	0.26			0.17		
(4)			0.14	±0.24	±0.13			±0.28		

TABLE 5.1.

B. Amino acids and intermediates of tricarboxylic-acid cycle and glycolysis.

	<u>CONCENTRATION (mM)</u>				
	<u>1.25</u>	<u>2.5</u>	<u>5</u>	<u>10</u>	<u>20</u>
Aspartate (6)	0.03 $\pm$ 0.07	-0.04 $\pm$ 0.12	-0.10 $\pm$ 0.14	-0.56 $\pm$ 0.21	
Glycine (4)	-0.03 $\pm$ 0.07	0.14 $\pm$ 0.13	0.06 $\pm$ 0.08	-0.04 $\pm$ 0.02	-0.03 $\pm$ 0.04
Serine (2)	0.22	0.03	-0.05	-0.45	-0.25
Citrate (5)	-0.17 $\pm$ 0.22	-0.08 $\pm$ 0.26	0.17 $\pm$ 0.35	0.04 $\pm$ 0.23	0.35 $\pm$ 0.35
Malate (4)	ZERO	-0.02 $\pm$ 0.04	0.17 $\pm$ 0.06	0.49 $\pm$ 0.12	0.61 $\pm$ 0.38
Succinate (6)	-0.41 $\pm$ 0.21	-0.24 $\pm$ 0.37	0.31 $\pm$ 0.29	0.39 $\pm$ 0.31	0.76 $\pm$ 0.48
Glyceraldehyde (2)	3.99	5.69		3.16	2.78
Sorbitol (4)				2.75 $\pm$ 0.22	
Xylitol (4)				1.80 $\pm$ 0.16	

TABLE 5.2.      Effects of added substrates on glycogen content of hepatocytes from 24h starved chickens.

Results are mean differences  $\pm$  SEM's due to 10 mM substrate addition from control glycogen contents ( $\mu\text{g}$  glucose - equivalents / $10^6$  hepatocytes) at specified incubation times from 3 experiments.

<u>Substrate</u>	<u>Incubation Time (min)</u>		
	<u>30</u>	<u>60</u>	<u>120</u>
Lactate	+0.12 $\pm$ 0.06	+0.12 $\pm$ 0.14	+0.01 $\pm$ 0.13
Pyruvate	-0.03 $\pm$ 0.10	+0.01 $\pm$ 0.11	+0.04 $\pm$ 0.03
Glycerol	-0.59 $\pm$ 0.31	-0.01 $\pm$ 0.03	+0.02 $\pm$ 0.13
Dihydroxyacetone	+0.20 $\pm$ 0.30	+0.07 $\pm$ 0.15	+0.25 $\pm$ 0.09
Fructose	+0.17 $\pm$ 0.02	+0.95 $\pm$ 0.17	+2.69 $\pm$ 0.67

TABLE 5.3. Additive effects of substrates on glucose production.

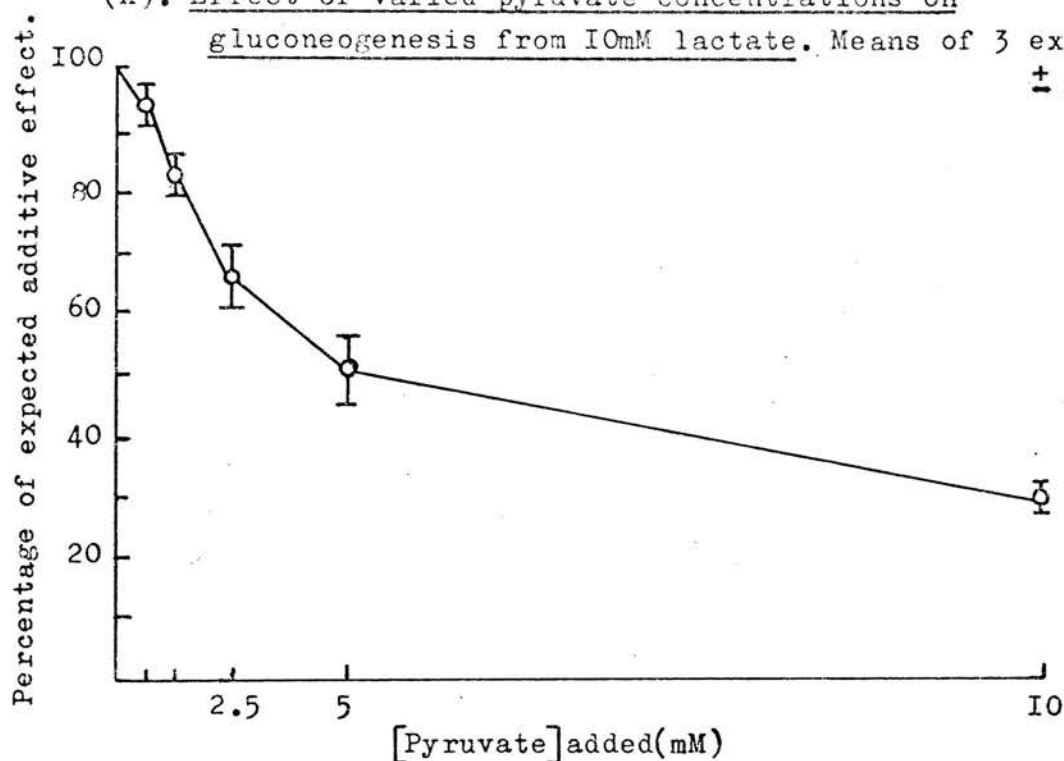
Hepatocytes from 24h starved chickens were incubated for 30 minutes with a single substrate (10 or 2 mM) or with mixtures of two substrates (each at 10 or 2 mM). Glucose production under each condition was measured and results are expressed as the percentage of the value expected from pure addition of production from each substrate. The results are means  $\pm$  SEM's with the number of experiments in parenthesis.

<u>1</u>	<u>SUBSTRATE</u>	<u>PERCENTAGE OF EXPECTED VALUE</u>	
	<u>2</u>	<u>10mM Substrate</u>	<u>2mM Substrate</u>
Lactate	Pyruvate(8)	31.6 $\pm$ 1.9	
"	Alanine(4)	85.3 $\pm$ 5.4	
"	Fructose(4)	72.8 $\pm$ 2.0	95.9 $\pm$ 2.4
"	Glycerol(4)	96.0 $\pm$ 3.8	
"	Dihydroxyacetone(5)	84.2 $\pm$ 2.5	94.8 $\pm$ 1.8
"	Hydroxypyruvate(4)	21.9 $\pm$ 2.5	
Pyruvate	Alanine(4)	74.7 $\pm$ 3.3	
"	Fructose(3)	96.1 $\pm$ 2.0	
"	Glycerol(7)	183.5 $\pm$ 18.0	
"	Dihydroxyacetone(4)	110.9 $\pm$ 5.7	
"	Sorbitol(4)	297.5 $\pm$ 15.9	
"	Xylitol(4)	178.8 $\pm$ 7.5	
Alanine	Fructose(4)	86.9 $\pm$ 2.8	
"	Glycerol(5)	152.9 $\pm$ 20.2	
"	Dihydroxyacetone(3)	97.0 $\pm$ 4.5	
Fructose	Glycerol(3)	102.8 $\pm$ 4.7	
"	Dihydroxyacetone(4)	75.8 $\pm$ 4.0	89.3 $\pm$ 3.6
Glycerol	Dihydroxyacetone(4)	82.2 $\pm$ 2.8	

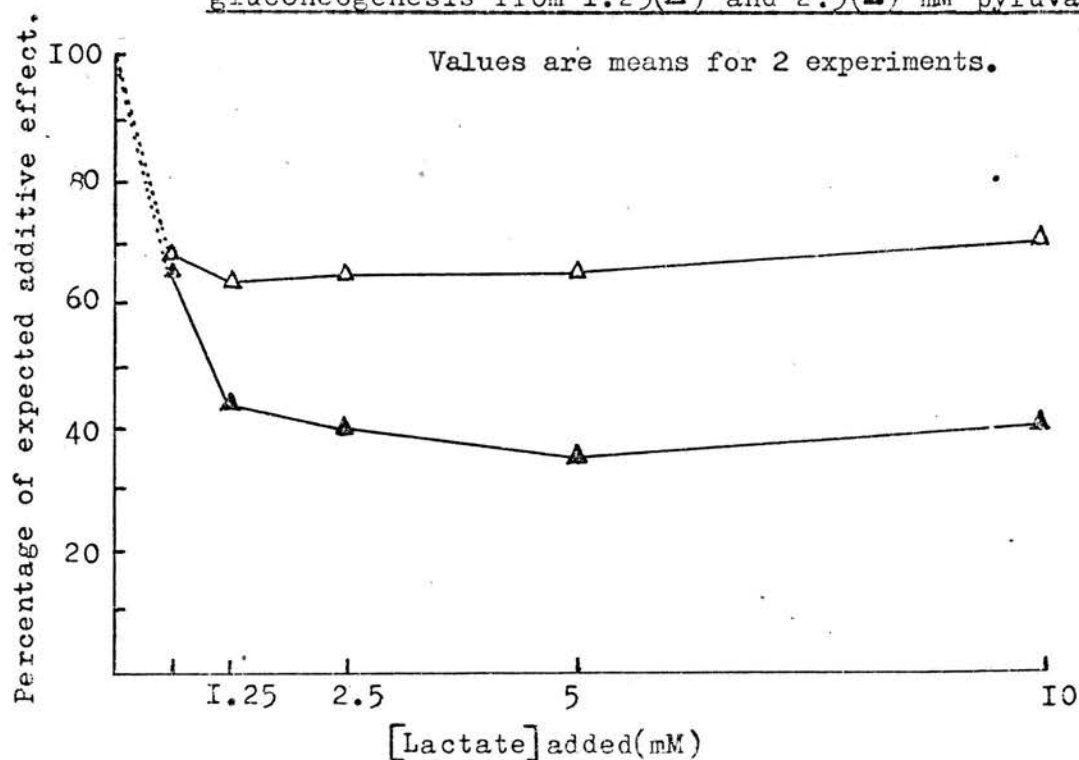
FIG. 5.4 Effect of lactate and pyruvate combinations on the rate of gluconeogenesis.

Hepatocytes from 24h starved chickens incubated for 30 min.

(A). Effect of varied pyruvate concentrations on gluconeogenesis from 10mM lactate. Means of 3 experiments  $\pm$  SEM.



(B). Effect of varied lactate concentrations on gluconeogenesis from 1.25( $\Delta$ ) and 2.5( $\Delta$ ) mM pyruvate.





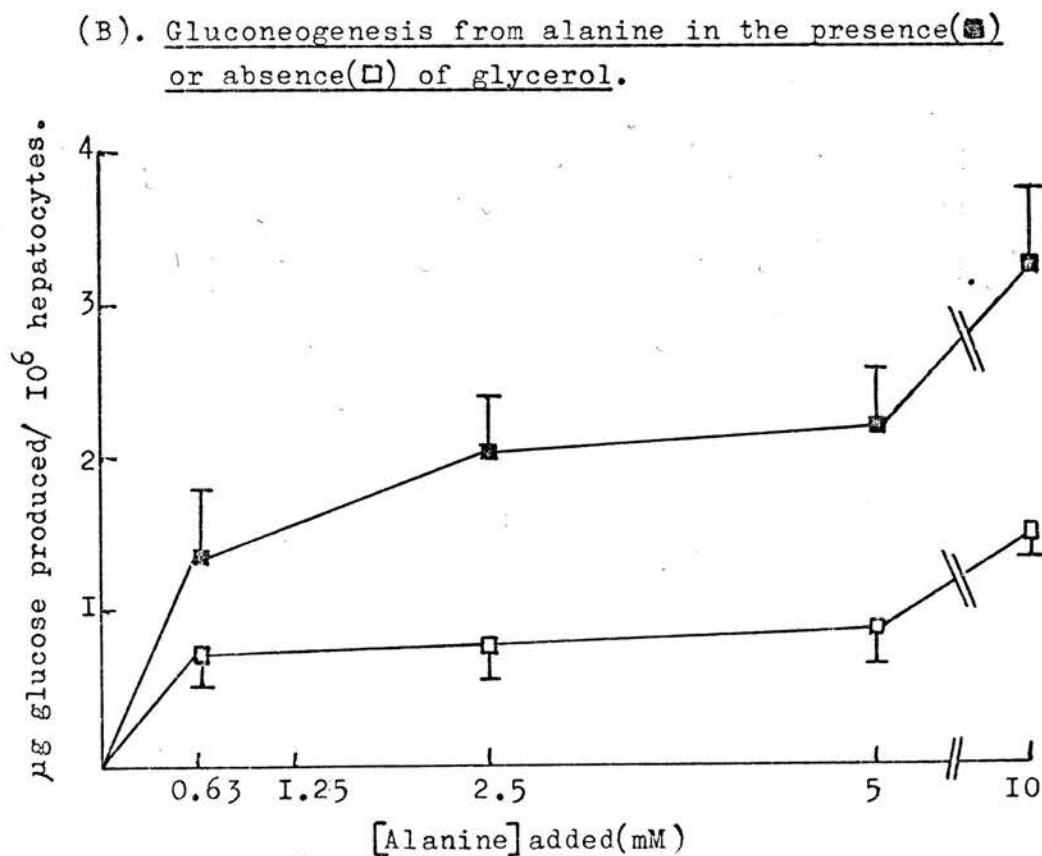
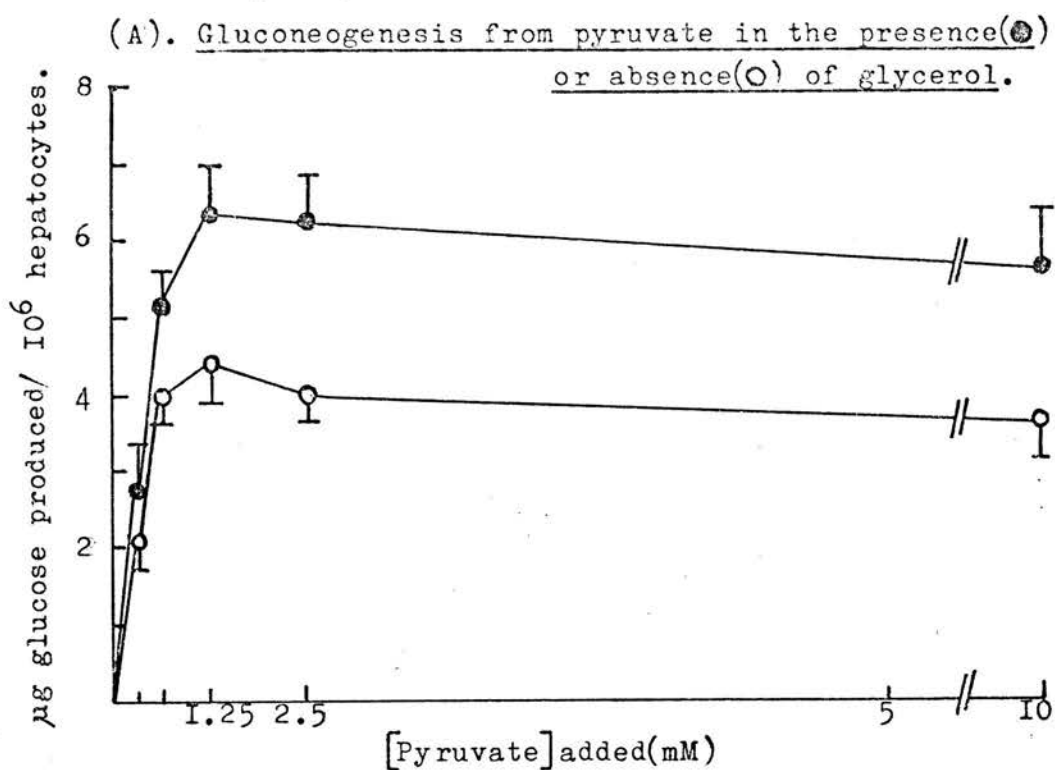
The difference between lactate and pyruvate was even more pronounced in hepatocytes from 48 hour starved chickens, while glycerol was still ineffective (Fig. 5.3).

Other amino acids (aspartate, glycine and serine) were poor glucose precursors and citric acid cycle intermediates (citrate, succinate and malate) only produced glucose at high concentrations (Table 5.1B). Hydroxypyruvate, xylitol and sorbitol produced only small amounts of glucose. Glyceraldehyde was a good precursor at low concentrations but specific interference of the glucose oxidase colour reaction prevented the accurate determination of gluconeogenesis at high concentrations (Table 5.1B).

Glycerol, DHA, pyruvate and lactate did not affect the glycogen content of hepatocytes during 2 hour of incubation (Table 5.2). Only fructose increased the glycogen content.

Glucose production in vivo will be complicated by interactions between several substrates. Several combinations of precursors do not have purely additive effects (Table 5.3). Net gluconeogenesis from mixtures of pyruvate and lactate was significantly less than expected by addition. The extent of inhibition of gluconeogenesis was dependent on the ratio of lactate/pyruvate (Fig. 5.4), and the characteristics of the inhibition suggest that the effect was probably due to disturbances of the equilibrium of the lactate dehydrogenase reaction. Alanine (10 mM) plus lactate caused inhibition of gluconeogenesis (Table 5.3). The inhibition was small and is probably due to pyruvate formed from alanine transamination. Combinations of lactate plus fructose, lactate/DHA or fructose/DHA (all at 10 mM) resulted in less than predicted rates of gluconeogenesis (Table 5.3). However, at high concentrations these

FIG.5.5 Effect of 10mM glycerol on gluconeogenesis from pyruvate and alanine by 24h starved chicken hepatocytes.  
Hepatocytes incubated for 30 min.



In each case values are the means  $\pm$  SEM from 3 experiments.

FIG. 5.6 Effect of ethanol concentration on gluconeogenesis.

Hepatocytes from 24h starved chickens incubated for 30 min and basal gluconeogenesis(O) and the stimulations caused by 10mM lactate( $\Delta$ ) and pyruvate( $\blacktriangle$ ) observed in the presence of ethanol.

Values are means  $\pm$  SEM from 3 experiments.

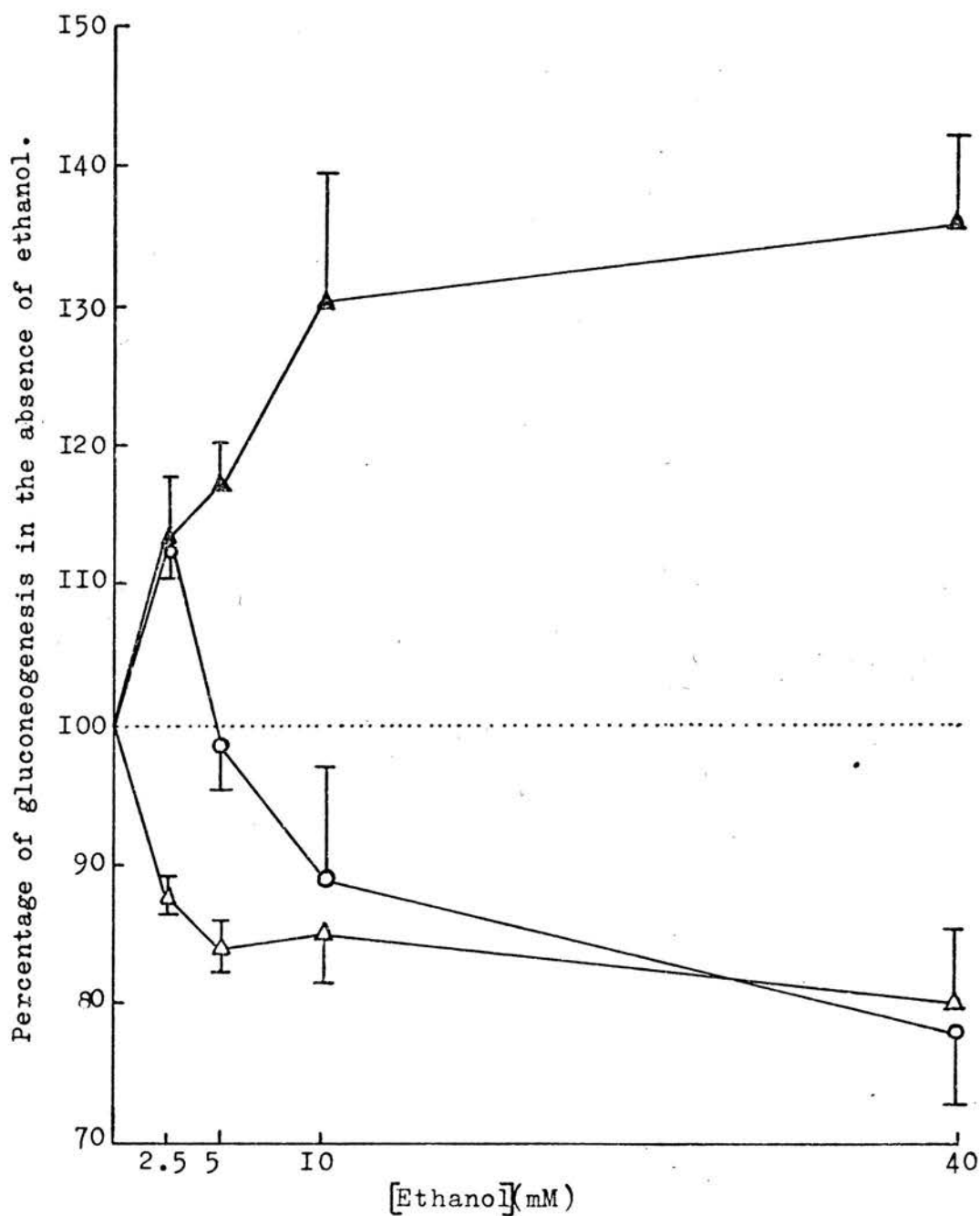
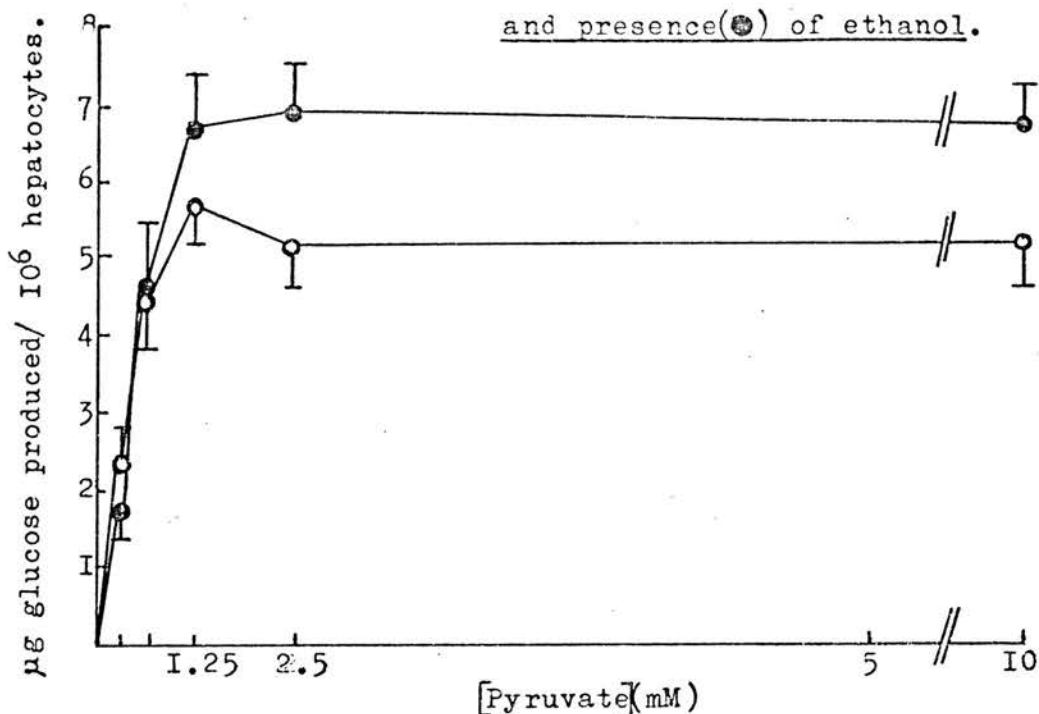


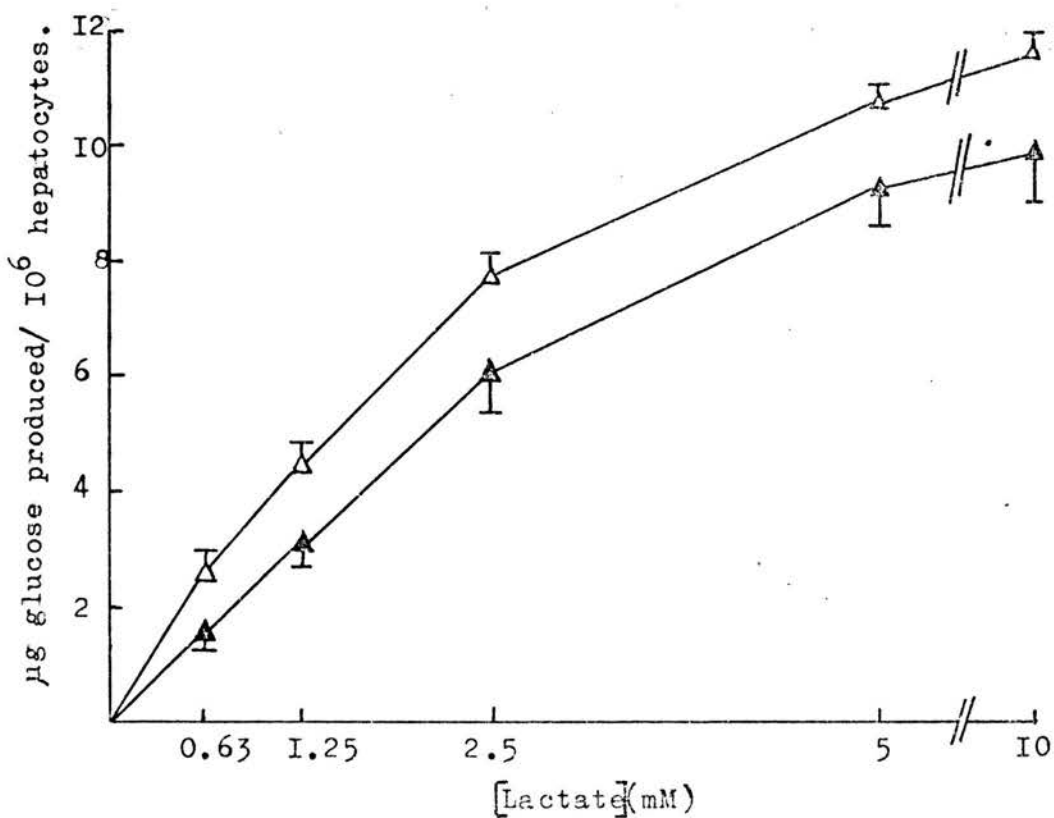
FIG. 5.7 Effects of 40mM ethanol on gluconeogenesis from varied concentrations of lactate and pyruvate.

Hepatocytes incubated for 30 min.

(A). Varied pyruvate concentrations in the absence(O) and presence(●) of ethanol.



(B). Varied lactate concentrations in the absence( $\Delta$ ) and presence( $\blacktriangle$ ) of ethanol.



In each case values are the means  $\pm$  SEM from 3 experiments.

precursors produced large quantities of glucose (Fig. 5.2) and it is possible that combinations of very active precursors may result in the saturation of gluconeogenesis. With their concentrations reduced to 2 mM, combinations of these precursors resulted in purely additive gluconeogenesis (Table 5.3). Thus the effects at 10 mM concentrations may have been due to saturation of steps in the gluconeogenic pathway.

Pyruvate plus either glycerol, sorbitol or xylitol resulted in greater than expected additive effects on gluconeogenesis (Table 5.3). The stimulation of glucose synthesis was related to the rate of gluconeogenesis by the reduced compound of the pair (Fig. 5.2 and Table 5.1). Thus, the extent of stimulation was in the order of sorbitol>xylitol>glycerol. Glycerol also potentiated gluconeogenesis from alanine (Table 5.3) presumably caused by the conversion of alanine to glucose occurring via pyruvate. The effect of glycerol on gluconeogenesis from pyruvate and alanine was dependent on the concentrations of pyruvate or alanine. (Fig. 5.5). The synergistic effect was maximal with 10 mM alanine or pyruvate.

#### Effects of ethanol and acetaldehyde

Ethanol inhibited gluconeogenesis from lactate and increased gluconeogenesis from pyruvate and the effects were maximal with 40 mM ethanol (Fig. 5.6). Hepatocytes incubated with ethanol were morphologically indistinguishable from control samples. The ethanol stimulation of gluconeogenesis from pyruvate occurred only with pyruvate concentrations above 0.4 mM, below that concentration gluconeogenesis was inhibited (Fig. 5.7). On the percentage basis, inhibition of gluconeogenesis from lactate was greatest at low lactate concentrations and decreased to a 25% inhibition with 10 mM lactate (Fig. 5.7).

TABLE 5.4. Effect of ethanol on gluconeogenesis from alanine, dihydroxyacetone, fructose, glycerol and hydroxypyruvate.

Hepatocytes from 24h chickens were incubated for 30 minutes with specified substrate concentrations in the presence or absence of ethanol (40 mM). Results are expressed as the means  $\pm$  SEM's of the number of experiments specified in parentheses.

<u>Substrate concentration (mM)</u>	<u>Ethanol effect - percentage of substrate only glucose production.</u>				
	<u>Alanine (3)</u>	<u>Dihydroxyacetone (3)</u>	<u>Fructose (3)</u>	<u>Glycerol (3)</u>	<u>Hydroxypyruvate (4)</u>
1	82.1 $\pm$ 14.1	67.0 $\pm$ 4.6	55.6 $\pm$ 11.3	56.4 $\pm$ 3.2	34.6 $\pm$ 10.3
2.5	83.3 $\pm$ 8.9	70.0 $\pm$ 4.7	66.5 $\pm$ 7.4	49.0 $\pm$ 2.1	
10	80.5 $\pm$ 14.5	69.5 $\pm$ 2.5	80.0 $\pm$ 5.8	51.3 $\pm$ 6.5	152.9 $\pm$ 16.3

TABLE 5.5.      Effects of acetaldehyde on gluconeogenesis.

Gluconeogenesis from glycerol or lactate (mM) was measured with hepatocytes from 24h starved chickens in the presence or absence of acetaldehyde. Values are expressed after subtraction of appropriate controls and are the means  $\pm$  SEM's of 3 experiments.

<u>Acetaldehyde (mM)</u>	<u><math>\mu\text{g}</math> glucose produced/<math>10^6</math> hepatocytes</u>	
	<u>Glycerol</u>	<u>Lactate</u>
0	1.33 $\pm$ 0.17	16.19 $\pm$ 2.61
1	2.05 $\pm$ 0.32	
5	2.46 $\pm$ 0.25	
10	1.12 $\pm$ 0.17	8.82 $\pm$ 0.92

TABLE 5.6. Effects of Adenosine on hepatocyte gluconeogenesis and adenylate content.

Hepatocytes from 24h starved chickens <sup>were</sup> incubated in the presence and absence of adenosine for 60 minutes and glucose production and adenylate content of hepatocytes were measured. Values are from 4 experiments and are expressed as means  $\pm$  SEM's.

A. <u>Gluconeogenesis</u>		<u><math>\mu</math>g glucose produced/<math>10^6</math> hepatocytes</u>	
<u>Adenosine added (mM)</u>	<u>Substrate added:</u>	<u>Glycerol (10mM)</u>	<u>Lactate (2mM)</u>
0		0.78 $\pm$ 0.09	3.10 $\pm$ 0.62
0.01		0.59 $\pm$ 0.06	3.27 $\pm$ 0.41
0.1		0.58 $\pm$ 0.06	0.92 $\pm$ 0.17
1.0		0.48 $\pm$ 0.11	0.61 $\pm$ 0.18

B. <u>Adenylate content</u>		<u>nmoles/<math>10^6</math> hepatocytes</u>		
<u>Adenosine added (mM)</u>		<u>ATP</u>	<u>ADP</u>	<u>AMP</u>
0		0.84 $\pm$ 0.18	0.44 $\pm$ 0.06	0.96 $\pm$ 0.13
0.01		0.88 $\pm$ 0.19	0.30 $\pm$ 0.18	0.92 $\pm$ 0.11
0.1		0.99 $\pm$ 0.13	0.65 $\pm$ 0.19	1.32 $\pm$ 0.25
1.0		0.91 $\pm$ 0.15	1.10 $\pm$ 0.31	1.76 $\pm$ 0.41
		<u><math>\Sigma</math> Adenylates</u>		<u>ATP/ADP</u>
		2.24 $\pm$ 0.30		1.91 $\pm$ 0.43
		2.09 $\pm$ 0.48		2.96 $\pm$ 0.61
		2.97 $\pm$ 0.59		1.51 $\pm$ 0.31
		3.77 $\pm$ 0.48		0.82 $\pm$ 0.35

Adenylate content was calculated in the absence of substrates



Ethanol inhibited gluconeogenesis from alanine, glycerol, DHA and fructose (Table 5.4). The inhibition was greatest with glycerol and least for alanine. The percentage inhibition with alanine, glycerol and DHA was similar whether the precursor was added to give a final concentration of 1 or 10 mM (Table 5.4). Increased fructose concentration decreased the extent of ethanol inhibition of gluconeogenesis. At low concentration (1 mM) gluconeogenesis from hydroxypyruvate was inhibited by ethanol whereas ethanol stimulated gluconeogenesis of 10mM hydroxypyruvate (Table 5.4).

Acetaldehyde (1 or 5 mM) stimulated glucose synthesis from glycerol (Table 5.5). At higher concentration (10mM), acetaldehyde inhibited gluconeogenesis from glycerol and lactate. Hepatocytes were morphologically similar whether incubated with or without acetaldehyde.

#### Effects of adenosine

Adenosine increased the ADP and AMP contents of hepatocytes in a concentration dependent manner (Table 5.6). As ATP was increased by a much smaller extent, adenosine decreased the ATP/ADP.AMP ratio. Gluconeogenesis from glycerol and lactate decreased as the ATP/ADP.AMP ratio fell (Table 5.6).

#### Glycerokinase activity

Liver homogenates phosphorylate glycerol at a constant rate during a 15 minutes incubation. The rate of phosphorylation was measured with high and low ATP and glycerol concentrations. Maximum rates were obtained with 1mM ATP and 4mM glycerol (Table 5.7). The omission of an ATP regenerating system and the presence of ADP and AMP at concentrations found for isolated chicken hepatocytes decreased



TABLE 5.7. Glycerokinase activity.

For details of assay see text. Results expressed on a wet weight basis.

A. Effect of ATP concentration on activity of liver homogenate glycerokinase.

ATP regenerating system present throughout a 15 minute incubation period with 4 mM Glycerol.

<u>ATP added (mM)</u>	<u>Units of glycerokinase activity /g liver.</u>
0.01	0.60
0.1	0.60
1.0	0.58
5.0	0.25

B. Maximum glycerokinase activities of liver and kidney homogenates.

Conditions as given in A. Maximum activity measured with 1 mM ATP values are the means of two experiments.

<u>Homogenate</u>	<u>Units of activity /g tissue</u>
Liver	0.63
Kidney	0.34

C. Effect of ADP and AMP on the glycerokinase activity of liver homogenates.

Assay had a 5 minute incubation period and buffers included 4 mM glycerol and 1 mM ATP in the presence or absence of 2 mM ADP and 2 mM AMP. ATP regenerating system was omitted from the buffers.

<u>ADP and AMP included</u>	<u>Units activity /g liver</u>
NO	1.07
YES	0.38

TABLE 5.8. Glucose production by liver slices from 24h starved chickens.

Liver slices (50-100 mg) were incubated for 1 hour as described in the text in the presence or absence of substrates (10 mM). Values are expressed after subtraction of appropriate controls and are the means  $\pm$  SEM's of 5 experiments.

<u>Substrate</u>	<u>Glucose production</u> <u>(mg /g wet weight /h)</u>
Lactate	1.59 $\pm$ 0.43
Pyruvate	0.51 $\pm$ 0.21
Alanine	0.16 $\pm$ 0.14
Glycerol	0.12 $\pm$ 0.11

glycerokinase activity to 36% of the maximum rate (Table 5.7). Under maximum conditions kidney homogenates phosphorylate glycerol at approximately half the rate of liver homogenates (Table 5.7).

#### Gluconeogenic activity of liver slices

Liver slices produce glucose from (in order of effectiveness) lactate, pyruvate, alanine and glycerol (Table 5.8). Although this ordering is similar to the isolated chicken hepatocyte, the rate of glucose production from 10 mM lactate ( $0.13 \mu\text{moles/g wet weight/min}$ ) was much less than that of isolated hepatocytes ( $0.86\text{--}1.36 \mu\text{moles/g wet weight/min}$ ).

#### DISCUSSION

The small rates of glucose production by hepatocytes from starved chickens are not balanced by glycogen degradation. As the changes involved are small the accuracy of determination may limit the ability to correlate glucose production with alteration in glycogen content. Thus it is difficult to determine the contribution of gluconeogenesis from endogenous sources to the basal glucose production of hepatocytes from starved chickens.

The constant rate of gluconeogenesis from lactate and the ineffectiveness of di- and tri-carboxylic acid precursors to synthesise glucose are indicators of the viability of isolated liver preparations (Ross, et al, 1967). Unlike fed chicken hepatocytes, there was no lag in glucose production from fructose and this may be due to the inability of fructose-1-phosphate to alter glucose production by inhibiting glycogen breakdown. The decreased rate of gluconeogenesis

from fructose between the first and second hours of incubation is due to an increased rate of glycogen deposition (Table 5.2). Glycogen synthesis by starved rat hepatocytes is low due to the substrate cycling with active phosphorylase and synthetase (Hers, 1976). In fed rat hepatocytes, phosphorylase is activated by anoxia during isolation (Hue, et al, 1975). If this also occurred in starved chicken hepatocytes, the synthesis of glycogen from fructose may be due to inhibition of phosphorylase by fructose-1-phosphate.

Gluconeogenesis occurs from lactate in the perfused chicken liver (Bickerstaffe, et al, 1970). Chicken liver slices synthesised smaller amounts of glucose when compared to the isolated hepatocyte but the effectiveness of precursors was similar (Table 5.8; Yarnell, et al, 1966; Bannister, et al, 1975). Injection of these precursors into starved chickens in vivo resulted in alterations in plasma concentration that were generally qualitatively similar to the effects on hepatocyte gluconeogenesis (Sarkar, 1971; Davison & Langslow, 1975). Lactate was more effective than pyruvate, alanine was the most effective of the amino acids and no substrates tested had any significant effect on liver glycogen content. In contrast to the isolated hepatocyte, glycerol and malate had effects on the plasma glucose concentration equivalent to lactate. Gluconeogenesis was also much greater from lactate than from pyruvate in pigeon liver (Krebs, et al, 1964; Söling, et al, 1973). The differences in gluconeogenesis effectiveness of precursors in vivo and in vitro will be discussed more fully in a later section of this chapter.

The rat has been widely used to study mammalian gluconeogenesis although <sup>whether</sup> it represents the typical mammal is debatable (Hanson, 1974). The gluconeogenic effectiveness of substrates on liver slices, perfused liver and isolated hepatocytes from rat may be ranked as follows:

fructose>DHA>lactate = pyruvate>alanine>glycerol>aspartate/tricarboxylic acid cycle intermediates (Ross, et al, 1967; Garrison & Haynes, 1973; Wagle & Ingebretson, 1975). Cornell & Filkins, 1974; { Rat liver preparations produce 0.75-0.90  $\mu$ moles glucose/g wet weight/min from 10 mM lactate. Thus the rate with isolated chicken hepatocytes (0.86-1.36  $\mu$ moles/g/min) is at least equivalent to that of rat preparations and may be significantly greater. Perfused pigeon liver synthesises 3  $\mu$ moles/g/min from 10 mM lactate (Söling, et al, 1973). Thus avian liver may have a greater capacity for lactate induced gluconeogenesis than rat. Although discrepancies exist, the general characteristics of gluconeogenesis in isolated chicken hepatocytes are similar to those of rat hepatocytes. The major differences are that in chicken lactate produces more glucose than pyruvate and that the rates of glucose synthesis from alanine and glycerol are much lower than in rat liver. The point of entry of substrates unto the gluconeogenic pathway of rat liver (see Fig. 4.1) is usually indicated by their maximal gluconeogenic abilities (Exton & Park, 1967 and 1969). Rate limiting reactions are indicated by large differences between precursors entering in similar parts of the pathway. On this basis rate limiting reactions occur in chicken hepatocytes between glycerol and DHA, alanine and pyruvate, lactate and pyruvate, and lactate and DHA. In the rat liver a control site exists between pyruvate and phosphoenolpyruvate and the difference in gluconeogenesis between lactate and DHA obtained for chicken hepatocytes may be due to a similar mechanism as in rat liver. As phosphoenolpyruvate carboxykinase is mitochondrial in chicken liver, the exact mechanism of control may not be identical. The limitation in gluconeogenesis from glycerol, alanine and pyruvate are discussed in greater detail in the next section of this chapter.

## GLUCONEOGENESIS OF ISOLATED CHICKEN HEPATOCYTES

When the effects of precursors on glucose production of isolated chicken hepatocytes are compared to results from other systems, significant differences emerge. In contrast to isolated rat liver preparations, isolated hepatocytes from starved chickens synthesise more glucose from lactate than from pyruvate. The rates of glucose production from lactate and pyruvate are equivalent with hepatocytes from fed chickens. Isolated hepatocytes show altered gluconeogenic effectiveness to chickens in vivo. Firstly, glycerol is much less effective in vitro than in vivo and secondly, to a lesser extent, this also applies for alanine.

### (1) Lactate and pyruvate

Lactate is known to be a more effective glucose precursor than pyruvate in isolated pigeon liver preparations (Krebs, et al, 1964; Söling, et al, 1973). These two precursors are separated metabolically only by lactate dehydrogenase and the state of the  $\text{NADH/NAD}^+$  ratio. The equilibrium of purified lactate dehydrogenase favours lactate synthesis and the lactate/pyruvate ratio of freeze clamped chicken liver suggests this also occurs in the chicken liver (Barratt, et al, 1974). The  $K_M$ 's for lactate and pyruvate with the purified enzyme are 6.7 and 0.16 mM respectively (Zewe & Fromm, 1962). The half-maximal rate of gluconeogenesis can be represented as an approximate " $K_M$ " for gluconeogenesis, and " $K_M$ 's" for lactate and pyruvate gluconeogenesis by starved chicken hepatocytes are 2.2 and 0.25 mM respectively (Fig. 5.2). The similarities of kinetic characteristics of lactate dehydrogenase and "gluconeogenesis" together with the observation that pyruvate decreases gluconeogenesis from lactate, suggests that the differences in effectiveness of lactate and pyruvate are related to lactate dehydrogenase action.

Pyruvate addition to pigeon liver homogenates results in lactate formation, a decrease in  $\text{NADH}/\text{NAD}^+$  ratio and in zero gluconeogenesis (Krebs & Hems, 1964). Conversely, lactate addition resulted in the formation of pyruvate, an increase in the  $\text{NADH}/\text{NAD}^+$  ratio and glucose synthesis. The differences in gluconeogenic effectiveness cannot be explained solely by the action of lactate dehydrogenase as the lactate dehydrogenase activity and the  $\text{NADH}/\text{NAD}^+$  ratio of chicken liver are similar to those of rat liver (Table 3.10; Williamson, *et al.*, 1967; Barratt, *et al.*, 1974). Other reactions of gluconeogenesis may interact with the pyruvate effects on lactate dehydrogenase to reduce the formation of glucose from pyruvate in chicken liver. For example, a decreased  $\text{NADH}/\text{NAD}^+$  ratio upon pyruvate addition may stimulate pyruvate dehydrogenase activity (Pettit, *et al.*, 1975) and lead to an increased flux of pyruvate carbon units to acetyl CoA rather than to phosphoenolpyruvate.

The cytosolic location of phosphoenolpyruvate carboxykinase in rat liver requires the passage of oxaloacetate produced from pyruvate or lactate into the cytosol for conversion to phosphoenolpyruvate and glucose (Fig. 4.1). Due to low mitochondrial permeability of oxaloacetate, it is converted to other four carbon compounds before diffusing into the cytosol. The carbon skeleton originating from pyruvate leaves the mitochondria as malate, whereas the skeleton from lactate exits as aspartate (Berry & Kun, 1972). The reconversion of malate to oxaloacetate will produce cytosolic NADH and as lactate previously produced NADH during conversion to pyruvate, both substrates will have produced cytosolic NADH for the reversal of the glyceraldehyde-3-phosphate dehydrogenase step essential for glucose synthesis.

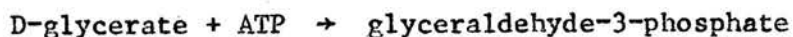


In chicken liver phosphoenolpyruvate carboxykinase is mitochondrial and carbon skeletons from both pyruvate and lactate leave the mitochondria as phosphoenolpyruvate in exchange for citrate or possibly ADP (Robinson, 1971; Shrago, et al, 1976; Söling & Kleineke, 1976). Thus without the involvement of malate in gluconeogenesis from pyruvate, NADH from endogenous sources will be required for the reversal of the glyceraldehyde-3-phosphate dehydrogenase reaction. Cytosolic NADH will not be limiting for lactate gluconeogenesis, but as pyruvate decreases NADH, high concentrations may result in the synthesis of so much lactate that NADH becomes limiting for the glyceraldehyde-3-phosphate dehydrogenase reaction. Thus the " $K_M$ " for gluconeogenesis from pyruvate will be lower than for lactate.

The effects of ethanol addition on gluconeogenesis also suggests that NADH becomes limiting (Fig. 5.7). Basal gluconeogenesis from pyruvate was maximal at 0.3-0.6 mM. At lower concentrations ethanol inhibited gluconeogenesis suggesting lactate production was increased. Only above 0.4 mM pyruvate does ethanol stimulate gluconeogenesis. Thus at lower concentrations of pyruvate endogenous NADH was probably sufficient for glucose synthesis, but above 0.4 mM pyruvate decreased the  $NADH/NAD^+$  ratio to the extent of limiting gluconeogenesis and added ethanol (NADH) can partly overcome this high substrate inhibition.

Although ethanol inhibited gluconeogenesis from lactate and stimulated gluconeogenesis from pyruvate, the rates of gluconeogenesis were not equivalent even at maximal ethanol concentrations (Fig. 5.6). The lactate/pyruvate ratio only reflects the ratio of  $NADH/NAD^+$  and alteration, specifically, of  $NADH/NAD^+$  by ethanol will effect a number of equilibria and not have as specific and potent action on the lactate/pyruvate ratio as might be predicted.

Hydroxypyruvate is converted to glucose by rat hepatocytes at 30% of the rate of gluconeogenesis from pyruvate and through reactions not involving phosphoenolpyruvate carboxykinase or the malate-oxaloacetate "shuttle" (Williamson & Ellington, 1975):



This precursor does not produce cytosolic NADH required for its conversion to glucose and ethanol increases gluconeogenesis from hydroxypyruvate presumably by stimulating glycerate formation due to increased NADH supply. In chicken hepatocytes pyruvate also produces three time more glucose than hydroxypyruvate (Table 5.1), and both precursors exhibit similar low  $K_M$ 's for gluconeogenesis. Ethanol also stimulated conversion of high concentrations of hydroxypyruvate to glucose (Table 5.4). Thus in both species the cytosolic NADH/NAD<sup>+</sup> ratio can be a major factor in determining the rate of precursor conversion to glucose.

The difference in gluconeogenesis between lactate and pyruvate in chicken liver is primarily caused by the equilibrium constant of lactate dehydrogenase and the subsequent effects of additions on the NADH/NAD<sup>+</sup> ratio. Rat liver does not distinguish between lactate and pyruvate due to the cytosolic location of phosphoenolpyruvate carboxykinase and the production of cytosolic NADH by mitochondrial-cytosolic "shuttle" systems.

With fed chicken hepatocytes lactate and pyruvate were equally effective in increasing glucose output. On the basis of increased oxygen consumption, lactate and pyruvate were metabolised to a greater

extent than glucose (Table 3.8; Heald, 1963). Much of the increased glucose production was probably due to substrate sparing effects on glucose oxidation. Glucose will be spared regardless of which substrate is oxidised instead of glucose.

Gluconeogenesis in isolated rat hepatocytes from lactate occurred after a lag phase which could be abolished by lysine addition (Johnson, et al, 1972; Krebs, et al, 1974). The lag was absent in chicken hepatocytes (Fig. 5.1). In the rat liver, carbons from lactate leave mitochondria as aspartate. As amino acids, and glutamate especially, are lost from hepatocytes during isolation (Krebs, et al, 1974), transamination of oxaloacetate to aspartate may be limited by low glutamate concentrations. Lysine transamination is low in rat liver but it may remove the lag by increasing the mitochondrial glutamate concentration. However, the effect must be extremely specific to lysine as no other amino acid had dramatic effects on the lag. As lactate conversion to glucose does not require aspartate exchange in the chicken liver, no lag would be expected. This provides another example of metabolic differences between rats and chickens resulting from the different intracellular location of phosphoenolpyruvate carboxykinase.

## (2) Glycerol

Glycerol and DHA enter the gluconeogenic pathway at closely related points, and their metabolism to glucose only differs by the nature of their phosphorylation and the oxidation of glycerol (Fig. 4.1). Glycerol is specifically phosphorylated by glycerokinase (E.C. 2.7.1.30) but DHA is phosphorylated either by glycerokinase or triokinase (E.C. 2.7.1.28) (Hers, 1962). Glyceraldehyde is metabolised by triokinase and its conversion to glucose (Table 5.1) shows the enzyme to be present in chicken

liver. As the permeability of both precursors into liver should not be limiting, the ineffectiveness of glycerol in relation to DHA in both fed and starved chicken hepatocytes must be due to limitations imposed by glycerokinase or  $\alpha$ glycerophosphate dehydrogenase activities.

The maximal glycerokinase activity of chicken liver homogenates (Table 5.7; Harding *et al.*, 1975) is only 30% of that reported for rat liver (Robinson & Newsholme, 1969) and the rate of glucose synthesis from glycerol in rat liver is seven times that of chicken hepatocytes (Ross, *et al.*, 1967). The isolation of hepatocytes decreased their total ATP content (see Chapter 3) Reduction of the ATP content of the assay buffer to 10  $\mu$ M (instead of 1-3 mM) in the presence of an ATP regenerating system did not decrease the homogenate glycerokinase activity (Table 5.7). However the presence of the ATP regenerating system will maintain high ATP/ADP.AMP ratios (even if the total ATP content was low), unlike the low ratios occurring in isolated chicken hepatocytes. With an assay buffer containing adenylates at a concentration found in isolated hepatocytes (ATP-1mM; ADP-2mM; AMP-2mM) and with no ATP regenerating system, glycerokinase activity was decreased by 64% (Table 5.7). If all the  $\alpha$ glycerophosphate produced with hepatocyte adenylate concentrations was to form glucose a maximum of 0.19  $\mu$ moles of glucose would be synthesised/min/g liver. Chicken hepatocytes produce a maximum of 0.07  $\mu$  moles glucose/min/g hepatocytes. This calculation does not take into account other metabolic fates of  $\alpha$ glycerophosphate or possible rate controlling steps prior to glucose production.

Incubation of hepatocytes with adenosine increased the total ATP content but resulted in an inhibition of gluconeogenesis from glycerol (Table 5.6). Adenosine presence increased ADP and AMP contents also to

the extent of decreasing the ATP/ADP.AMP ratio. Thus it appears probable that the ATP content does not control the rate of glycerol phosphorylation by isolated hepatocytes, but that as determined for the enzyme in homogenates it is the ratio of ATP/ADP.AMP that influences glycerol metabolism. The low ATP/ADP.AMP ratio of isolated hepatocytes probably inhibits glycerol conversion to glucose and explains the difference in glucose production from glycerol in vivo and in vitro.

Ethanol inhibition and acetaldehyde stimulation of gluconeogenesis from glycerol, along with the increased glucose synthesis when pyruvate or alanine (cytosolic  $\text{NAD}^+$  generators) are added with glycerol, suggested that the conversion of  $\alpha$ glycerophosphate to glucose may be limited at the level of  $\alpha$ glycerophosphate dehydrogenase by the  $\text{NADH}/\text{NAD}^+$  ratio. At the maximum, acetaldehyde increased gluconeogenesis from glycerol 250% and 0.18  $\mu\text{moles}$  of glucose were then synthesised/min/g hepatocytes. Bearing in mind the assumptions made, this is extremely close to the predicted rate from the maximal glycerokinase activity obtained with an ATP/ADP.AMP ratio similar to that measured in isolated chicken hepatocytes.

Although the  $\text{NADH}/\text{NAD}^+$  ratio of chicken liver in vivo may be regulated by the presence of pyruvate and alanine in the extracellular fluid, and hence modify glucose synthesis from glycerol, it is unlikely to be a major factor responsible for the low rate of gluconeogenesis from glycerol in vitro. The low ATP/ADP.AMP ratio of isolated hepatocytes is the main factor. However, at the maximum only 0.54  $\mu\text{moles}$  of glucose could be synthesised per min/g liver from glycerol (zero ADP and AMP), whereas lactate produces 0.8-1.2  $\mu\text{moles}$  glucose/min/g hepatocytes. Yet glycerol is as effective as lactate on plasma glucose concentration in vivo. The presence of glycerokinase in kidney homogenates with 50% of liver activity (Table 5.8) will permit gluconeogenesis from glycerol by the kidney.

However, as the kidney may synthesise glucose from lactate at a rate equivalent to liver, glycerol metabolism by the kidney cannot fully explain the discrepancy. The effectiveness of lactate in vivo may be reduced by peripheral utilisation lowering the actual hepatocyte lactate concentration, whereas glycerol might not be so easily metabolised.

### (3) Alanine

Increased alanine release and turnover from protein degradation in peripheral tissues during starvation has been interpreted as evidence for its importance in gluconeogenesis (Felig, et al, 1970). Of the amino acids injected into chickens in vivo, it caused the most significant increase in plasma glucose concentration (Davison & Langslow, 1975).

In the perfused rat liver, the rate of transamination of alanine to pyruvate limits its further metabolism (Mallette, et al, 1969). The activity of alanine aminotransferase (E.C. 2.6.1.2) in chicken liver is only 1% of that in rat liver (Sarkar, 1971), and attempts to measure its activity in isolated chicken hepatocytes during my project gave unreliable results due to extremely low activities. The low rate of glucose production from alanine by fed and starved chicken hepatocytes compared to rat hepatocytes can be explained by differences in the transaminase activity.

The extent of inhibition of gluconeogenesis from a combination of lactate and alanine, when compared to the inhibition with lactate and pyruvate together, permits an estimate of the amount of pyruvate produced from alanine and hence of transaminase activity. Thus 10 mM alanine has an effectiveness equivalent to the addition of 0.6 mM pyruvate. The inhibition of glucose synthesis from 10 mM alanine by ethanol may thus be

explained by the mechanism of ethanol inhibition of gluconeogenesis from low (less than 0.6 mM) concentrations of pyruvate.

The effects of alanine in vivo cannot be explained by the gluconeogenic capacity of chicken liver in vitro over two hour incubations. Although kidney may be an important site of alanine gluconeogenesis, other factors in vivo may elevate the hepatic conversion of alanine to glucose. Alanine reduces the cytosolic NADH/NAD<sup>+</sup> ratio of rat liver (Williamson, et al, 1969a) and glycerol conversion to glucose by isolated chicken hepatocytes is stimulated by alanine and acetaldehyde (Tables 5.3 and 5.6). Injection of alanine in vivo may not only result in the conversion of alanine carbon skeletons into glucose but also stimulates the rate of gluconeogenesis from circulating glycerol. The net effect would be a rate of gluconeogenesis greater than that from in vitro studies. Amino acid uptake is stimulated in the presence of hormones (Park & Exton, 1972) and their action may influence the rate of gluconeogenesis from alanine in vivo (Chapter 6).

#### SIGNIFICANCE OF RESULTS FROM ISOLATED HEPATOCYTES ON THE CONTROL OF PLASMA GLUCOSE IN VIVO

Hepatocytes in vivo will be constantly bathed in plasma containing a wide variety of metabolites. The combination of several precursors during incubations with isolated hepatocytes allows the investigation of interactions that might also occur in vivo. This study indicates the necessity of accounting for the interactions before directly extrapolating results obtained in vitro to the situation in vivo. On the basis of the lack of stimulation of oxygen consumption by liver slices (Heald, 1963), sorbitol is presumed not <sup>to</sup> be metabolised by chicken (Hazelwood, 1972).



Xylitol metabolism to glucose requires enzymes of the pentose phosphate pathway, and those required (transketolase, transaldolase) are stated to be absent from chicken liver (Duncan & Common, 1967). These two metabolites each only give rise to small amounts of glucose synthesis (Table 5.1). In the presence of pyruvate gluconeogenesis was greatly stimulated (Table 5.3). The metabolism of xylitol and sorbitol requires dehydrogenation and the decreased  $\text{NADH/NAD}^+$  ratio from pyruvate addition may increase their rate of oxidation. Thus xylitol and sorbitol may be metabolised to a greater extent in vivo than in vitro, and the role of the pentose phosphate cycle in chicken liver in vivo requires further evaluation.

This study has only investigated the interactions of a relatively few glucose precursors. Apart from the complexity of their interactions in vivo, fatty acids and amino acids will influence gluconeogenesis, either by direct effects or by sparing the oxidation of specific precursors. Over and above the substrate interactions on gluconeogenesis, there is the influence of circulating hormones to be taken into account.

Isolated hepatocytes show qualitative similarities to effects on plasma glucose in vivo and offer an ideal system for studying the mechanism of gluconeogenesis. The hepatocytes synthesised glucose from alanine, pyruvate, lactate and glycerol and the rates of synthesis were responsive to changes of precursor concentrations in the physiological (0.2-4 mM) range. However the isolation of hepatocytes may result in a preparation unsuitable for studying some aspects of gluconeogenesis. Only glycerol gluconeogenesis appears to be impaired in a manner not explained by effects of other plasma constituents. Alteration of adenylates during hepatocyte isolation is specifically responsible for glycerol ineffectiveness. The possibility that other precursors may be



inactive due to hepatocyte degeneration can not be neglected.

Degradation of hepatocyte glycogen will rapidly compensate for decreases in plasma glucose. Although glycogen is resynthesised in vivo, net synthesis was not obtained in isolated hepatocytes probably due to activation of phosphorylase and inactivation of synthetase during isolation. Depression of phosphorylase activity by manipulation of ionic conditions may result in synthetic ability. This does, however, make them ideal for studying glucose production over short time-course experiments.

As shown for glycerokinase activity, kidney may be significant in glucose synthesis and little information is available on its role in the control of plasma glucose in chicken. In the mouse, kidney synthesises glucose preferentially from specific amino acids, whereas liver utilises others for gluconeogenesis (Krebs, et al, 1966). The importance of kidney in chicken deserves further study.

#### SUMMARY

Hepatocytes isolated from starved chickens synthesised glucose from a wide range of precursors. All the glucose was released into the surrounding medium and only fructose caused the synthesis of significant amounts of glycogen. The lack of glycogen synthesis was suggested to be due to high phosphorylase activity.

The gluconeogenic effectiveness of specific precursors was similar to their effectiveness on elevating plasma glucose concentration in vivo, except for low rates of gluconeogenesis from alanine and glycerol. The inability to convert glycerol into glucose has been shown to be due to

a decrease of hepatocyte ATP/ADP.AMP ratio during isolation and the inhibitory effect of ADP and AMP on glycerokinase activity.

The ordering of effectiveness of the precursors (fructose>DHA>lactate>pyruvate>alanine>glycerol>di- and tricarboxylates) is largely similar to that of rat hepatocytes. In rat hepatocytes, however, pyruvate is equivalent to lactate and alanine is much more effective. The low activity of alanine can be explained by the low activity of alanine aminotransferase in chicken liver. The lack of mitochondrial-cytosolic shuttle systems due to the mitochondrial location of phosphoenolpyruvate carboxykinase is responsible for the low rate of pyruvate gluconeogenesis by chicken liver.

The addition of these precursors in combination has permitted the determination of some of the substrate level interactions that may occur in vivo. Some of the mechanisms involved in these interactions were investigated by the alteration of the  $\text{NADH/NAD}^+$  ratio of hepatocytes. Particularly significant was the synergistic effect of gluconeogenesis of glycerol plus pyruvate or alanine which will be of physiological importance.

Isolated chicken hepatocytes have proven to be a suitable system for studying gluconeogenesis and its mechanisms in vitro. However, the present study has shown that the complexities of plasma interactions makes it impossible to directly correlate in vitro studies with effects in vivo and that great care must be taken in extrapolating results.

## CHAPTER 6

### STIMULATION OF HEPATIC GLUCOSE

#### PRODUCTION BY GLUCAGON

### INTRODUCTION

The injection of glucagon into chickens increases the plasma glucose concentration (Heald, et al, 1965; Grande, 1968; Langslow, et al, 1970). In mammals the action of glucagon on liver elevates glucose production from glycogenolysis and gluconeogenesis (for review see Park & Exton, 1972) and is largely responsible for the increased plasma glucose concentration. The effects are mediated through increased cyclic-AMP production, and the injection of glucagon into chickens in vivo raises the liver cyclic-AMP content (Fröhlich, & Marquardt, 1972).

The mechanism of glucagon action on the chicken liver has not previously been investigated and isolated hepatocytes were used to investigate its effects on glycogenolysis and gluconeogenesis. By comparing the effects of glucagon on several gluconeogenic precursors, the sites of action of glucagon can be observed. Although isolated hepatocytes offer several advantages for studying hormonal effects over other techniques in vitro, proteolytic contaminants in collagenase may affect hormonal sensitivity (Kono & Barham, 1967; Johnson, et al, 1962). The sensitivity of hepatocytes to glucagon is thus also a useful indicator of the quality of preparations.

### METHODS

Glucagon was dissolved in N/300 HCl to give a stock solution of 1 mg/ml and working strength dilutions were prepared freshly each day

with KRB containing 2% BSA.

The effects of glucagon are expressed as the percentage of control value or as the extra quantity of glucose produced (or lost) per  $10^6$  hepatocytes in the presence of glucagon. Since variability occurs between the sensitivity of batches of hepatocytes, the variation will result in decreased significance of glucagon effects. To illustrate the significance of glucagon effects, single representative experiments (of at least three qualitatively similar experiments) are presented in certain instances.

Receptor damage may be caused by proteolytic contaminants in collagenase. Midway through my project the properties of the collagenase obtained from Boehringer altered and this resulted in hepatocytes with greatly reduced glucagon sensitivity (see Chapter 2). The preparation of hepatocytes by method B resulted in improved glucagon sensitivity and all results described in this chapter were obtained from cells prepared by method B.

## RESULTS

### Glucagon effects on fed chicken hepatocytes

Glucagon increased glucose production at all time intervals during two hours incubation (Fig. 6.1.A). The increment was constant at all time intervals. Increased glycogen loss accounted for the alteration in glucose production and the glucagon effect on glucose production was greater with hepatocytes with high initial glycogen content (Fig. 6.1.B). Hepatocytes isolated from fed chickens early in the morning had low glycogen content (see Chapter 4) and glucagon had lower effects on

FIG. 6.I Effect of incubation time on stimulation of glycogenolysis by glucagon.

Results of 2 individual experiments expressed as means  $\pm$  SD.

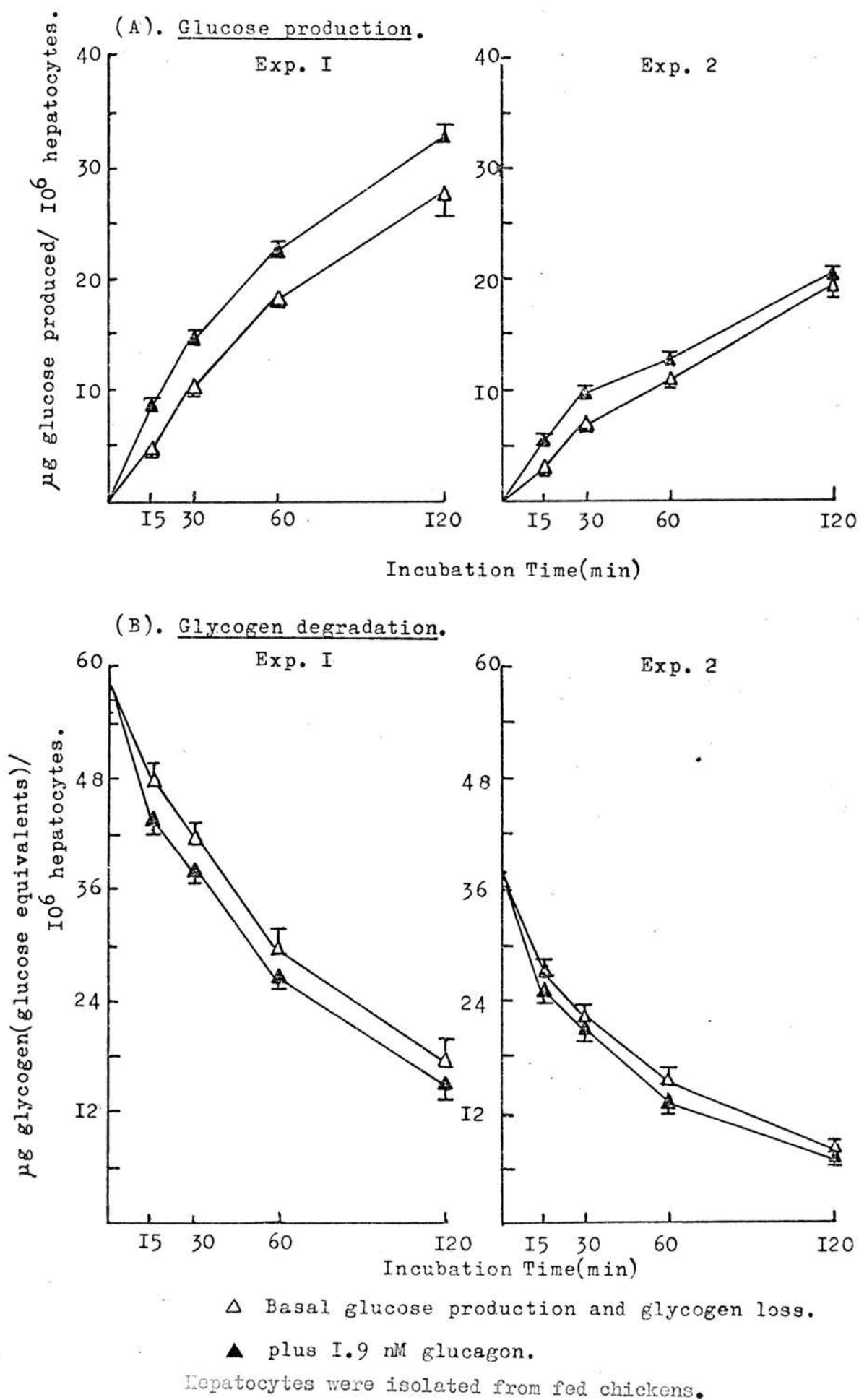


FIG. 6.2 Maximal stimulation of glucose production by glucagon and dibutyryl cAMP.

Hepatocytes from fed chickens incubated for 30 min in the presence and absence of glucagon or dibutyryl cAMP. Values are means  $\pm$  SEM's for 3 experiments.  $\Delta$  Glucagon ;  $\bullet$  Dibutyryl cAMP

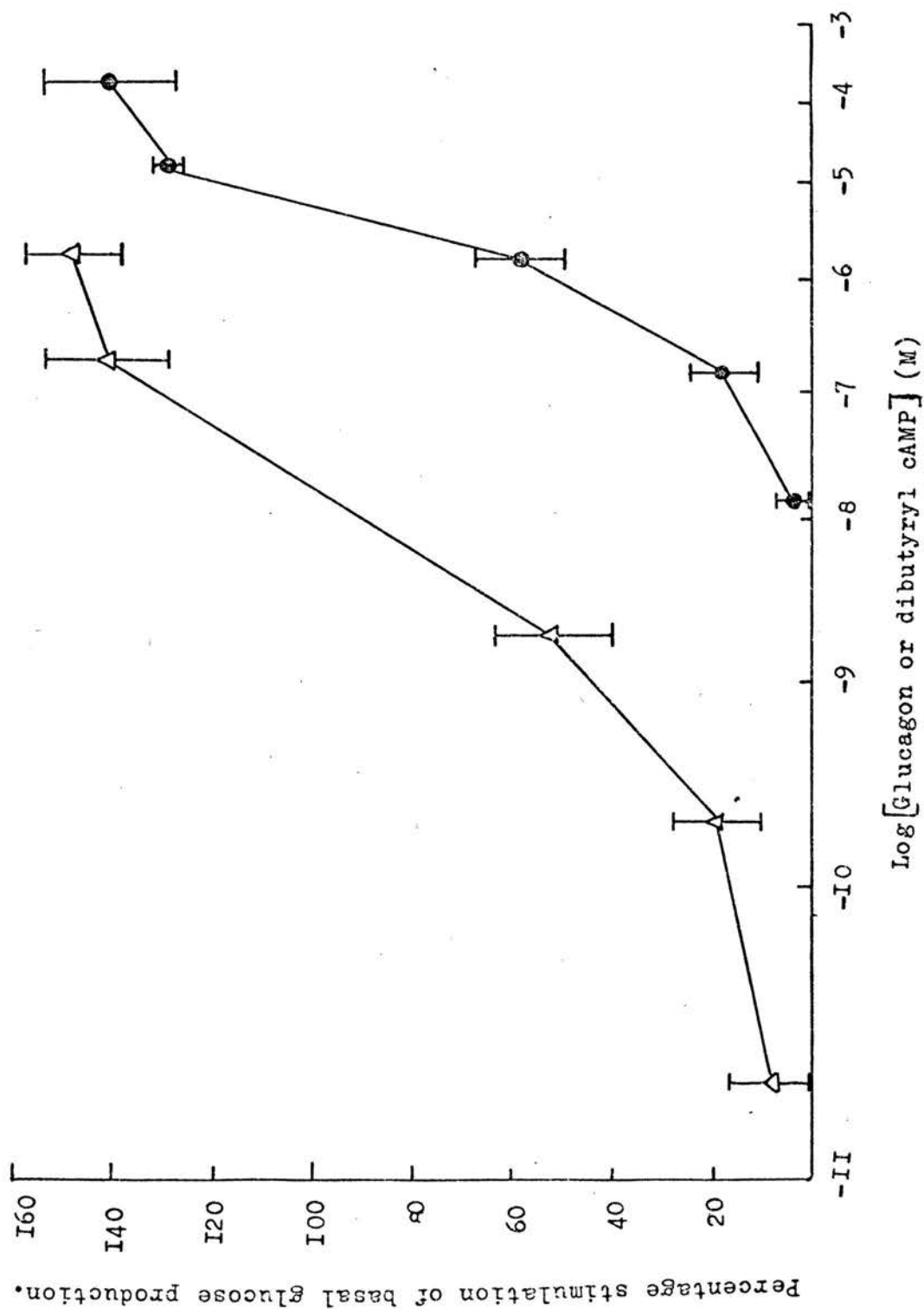


TABLE 6.1. Effects of glucagon concentration on glycogen content of hepatocytes isolated from fed chickens.

Hepatocytes were incubated for 30 minutes and then glycogen content was measured in the presence or absence of glucagon.

Results are means  $\pm$  SEM's for 3 experiments.

<u>Glucagon</u> <u>concentration (M)</u>	<u><math>\mu</math>g glycogen</u> <u>(glucose-equivalents)</u> <u>10<sup>6</sup> hepatocytes</u>
ZERO	39.31 $\pm$ 1.54
1.9x10 <sup>-11</sup>	38.85 $\pm$ 1.71
1.9x10 <sup>-10</sup>	38.37 $\pm$ 1.93
1.9x10 <sup>-9</sup>	36.64 $\pm$ 1.74
1.9x10 <sup>-7</sup>	31.59 $\pm$ 1.24
1.9x10 <sup>-6</sup>	33.62 $\pm$ 1.74



glucose production. Maximum glucagon effects were only obtained with hepatocytes containing at least 30-50  $\mu\text{g}$  glycogen (glucose-equivalents) per  $10^6$  hepatocytes. All subsequent experiments were of 30 minutes duration as glucose production was highest and the glucagon effects greatest during the first 30 minutes of incubation.

Maximal stimulation of glucose production occurred with  $10^{-6}$  M glucagon and significant effects were obtained with  $10^{-10}$  M (Fig. 6.2). Dibutyryl cyclic AMP mimicked the effects of glucagon and half-maximal effects were elicited by  $9.3 \times 10^{-9}$  M glucagon and  $2.0 \times 10^{-6}$  M dibutyryl cyclic AMP. Glycogen loss was dependent on the glucagon concentration (Table 6.1) and mirrored the effects on glucose production.

Caffeine and, to a lesser extent, theophylline inhibited endogenous glycogenolysis and largely prevented glucagon stimulation (Fig. 6.3). In the presence of caffeine, the glucagon stimulation of cyclic AMP accumulation was potentiated (D.R. Langslow, personal communication).

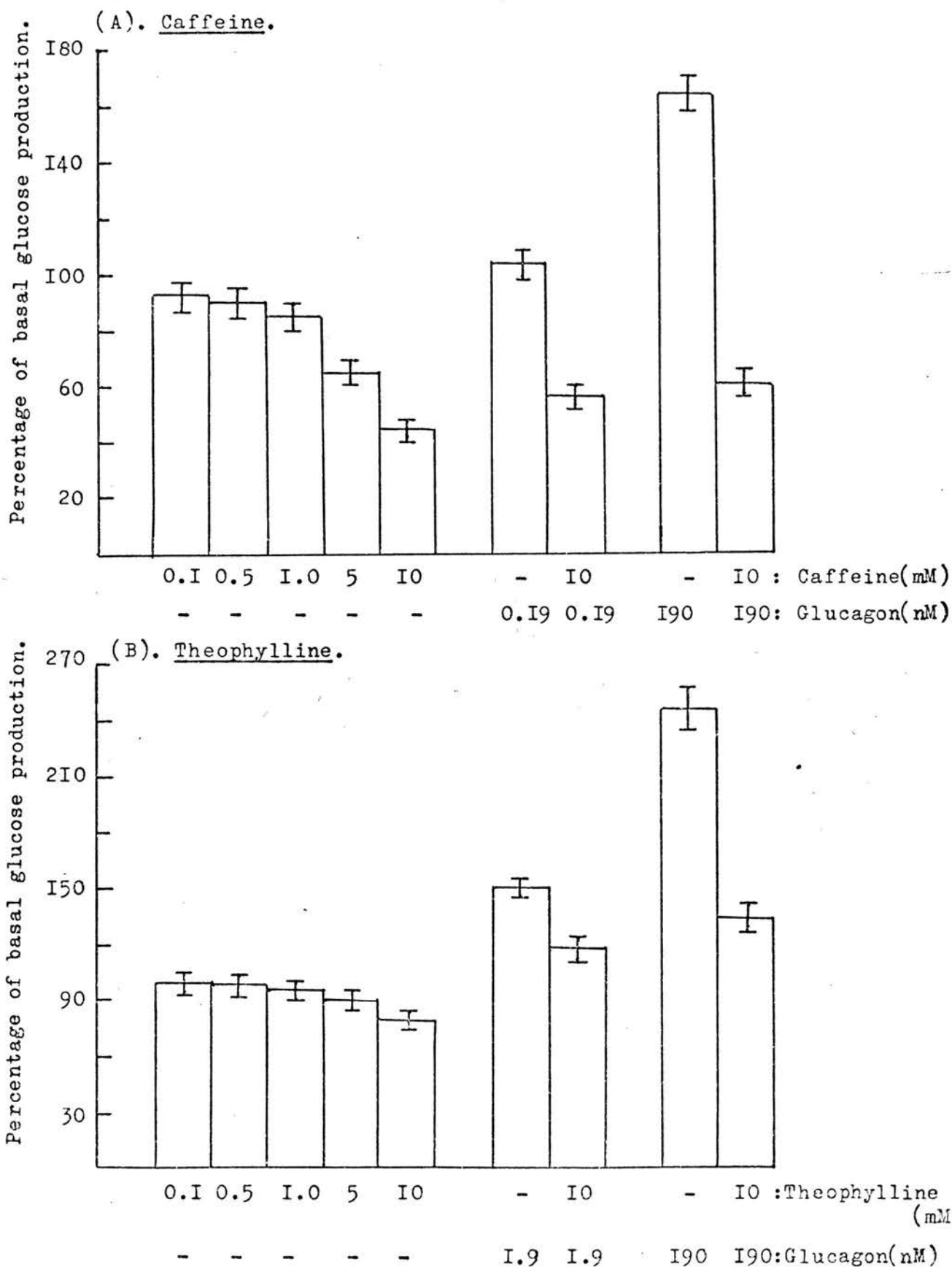
#### Glucagon effects on starved chicken hepatocytes

The extent of gluconeogenesis in fed chicken hepatocytes from added precursors can only be measured with radioactively labelled precursors. Thus the actions of glucagon on gluconeogenesis were investigated with hepatocytes from 24 hour starved chickens where glycogen was not involved.

Glucagon stimulated endogenous glucose production and gluconeogenesis from lactate (Fig. 6.4). As with fed chicken hepatocytes the stimulation was obvious at the first time interval and there was no further increase in the amount of additional glucose produced. There was no detectable

FIG. 6.3 Effects of caffeine and theophylline on glucose production from glycogen by fed chicken hepatocytes.

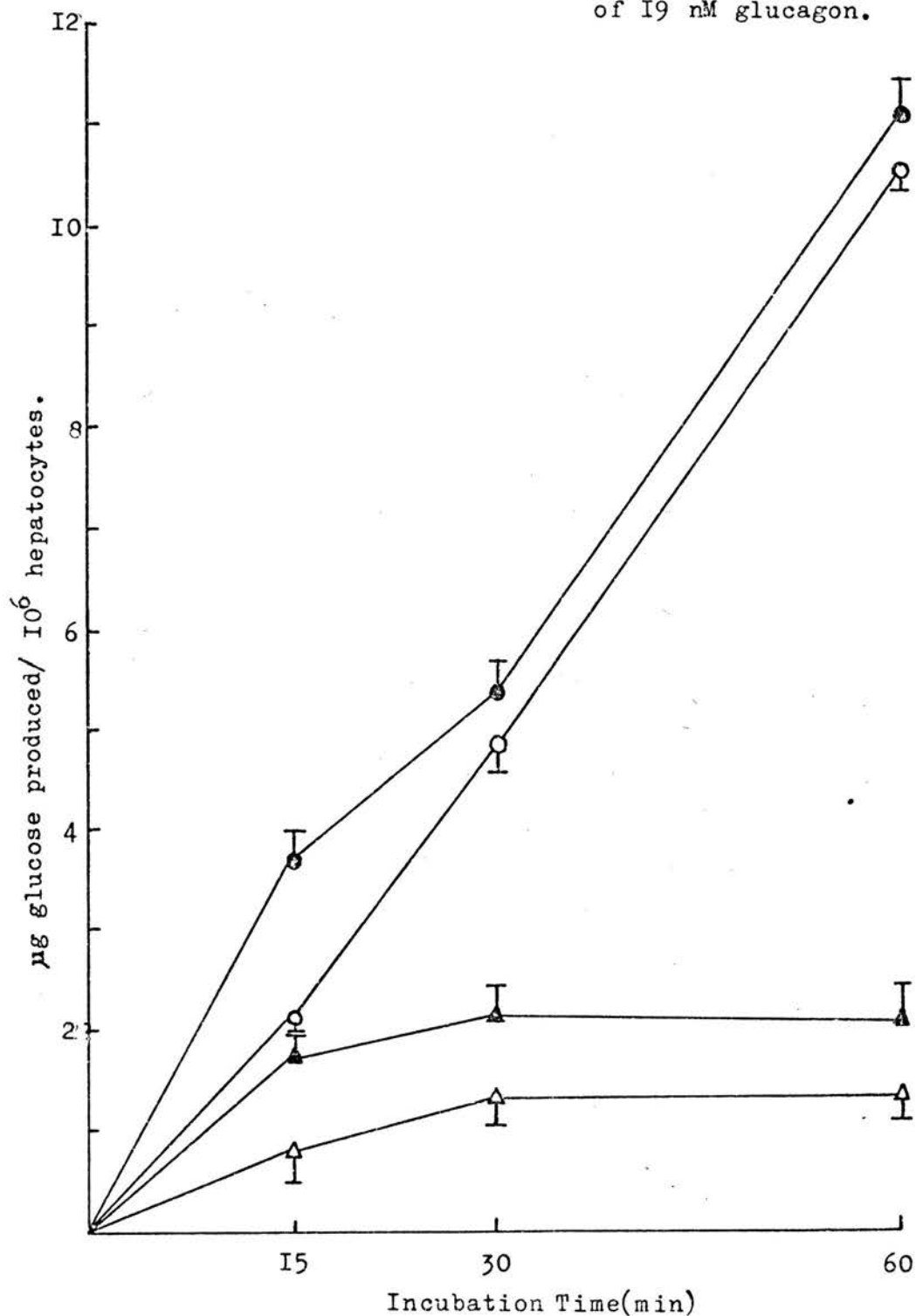
Hepatocytes incubated with or without additions for 30 min.



Results are the means from a single representative experiment  $\pm$  SD.

FIG. 6.4 Effect of incubation time on stimulation of gluconeogenesis by glucagon.

The endogenous gluconeogenesis of hepatocytes from 24h starved chickens was measured in the absence( $\Delta$ ) and presence( $\blacktriangle$ ) of 19nM glucagon. The values were used as appropriate basals for gluconeogenesis from 10mM lactate in the absence( $\circ$ ) and presence( $\bullet$ ) of 19 nM glucagon.



Results are the means from a single representative experiment  $\pm$  SD.



alteration in glycogen content with glucagon addition.

Gluconeogenesis from lactate was stimulated at all concentrations (1.25-20 mM) studied (Table 6.2). The percentage stimulation was similar for all but the highest concentration. Maximal effects of glucagon were measured with 2.5 mM lactate, which also ensured against substrate saturation effects (Fig. 6.5). Maximal stimulation was 34% and  $7.7 \times 10^{-10}$  M glucagon resulted in half-maximal stimulation. Dibutyryl cyclic AMP mimicked the glucagon effect and gave a similar maximum (Fig. 6.5).

Glucagon also stimulated gluconeogenesis from pyruvate, alanine, glycerol, fructose and DHA (Table 6.3) and maximal effects occurred with 0.31 mM pyruvate, 1 mM glycerol and 1 mM alanine (Table 6.2). Gluconeogenesis from glycerol was stimulated to the greatest extent, while the percentage stimulations for the other substrates were similar. Glucose synthesis from fructose and DHA is also stimulated by dibutyryl cyclic AMP (Table 6.3).

## DISCUSSION

Although the sensitivity of the effects was variable from day to day due, presumably, to the extent of damage occurring to receptors during isolation, glucagon stimulated glycogenolysis and gluconeogenesis. The activation of these processes occurred with glucagon concentrations similar to peripheral plasma concentrations ( $3 \times 10^{-10}$  M) reported for starved chickens (Krug, et al., 1976). The effects on both these processes were maximal after 15 minutes incubation (Figs 6.1 and 6.4) and no further stimulation occurred. As the half-life of glucagon in

FIG. 6.5 Maximal stimulation of gluconeogenesis by glucagon and dibutylryl cAMP.

Hepatocytes from 24h starved chickens incubated for 30 min in the presence or absence of glucagon( $\Delta$ ) or dibutylryl cAMP( $\bullet$ ), and gluconeogenesis from 2.5 mM lactate measured after subtraction of appropriate

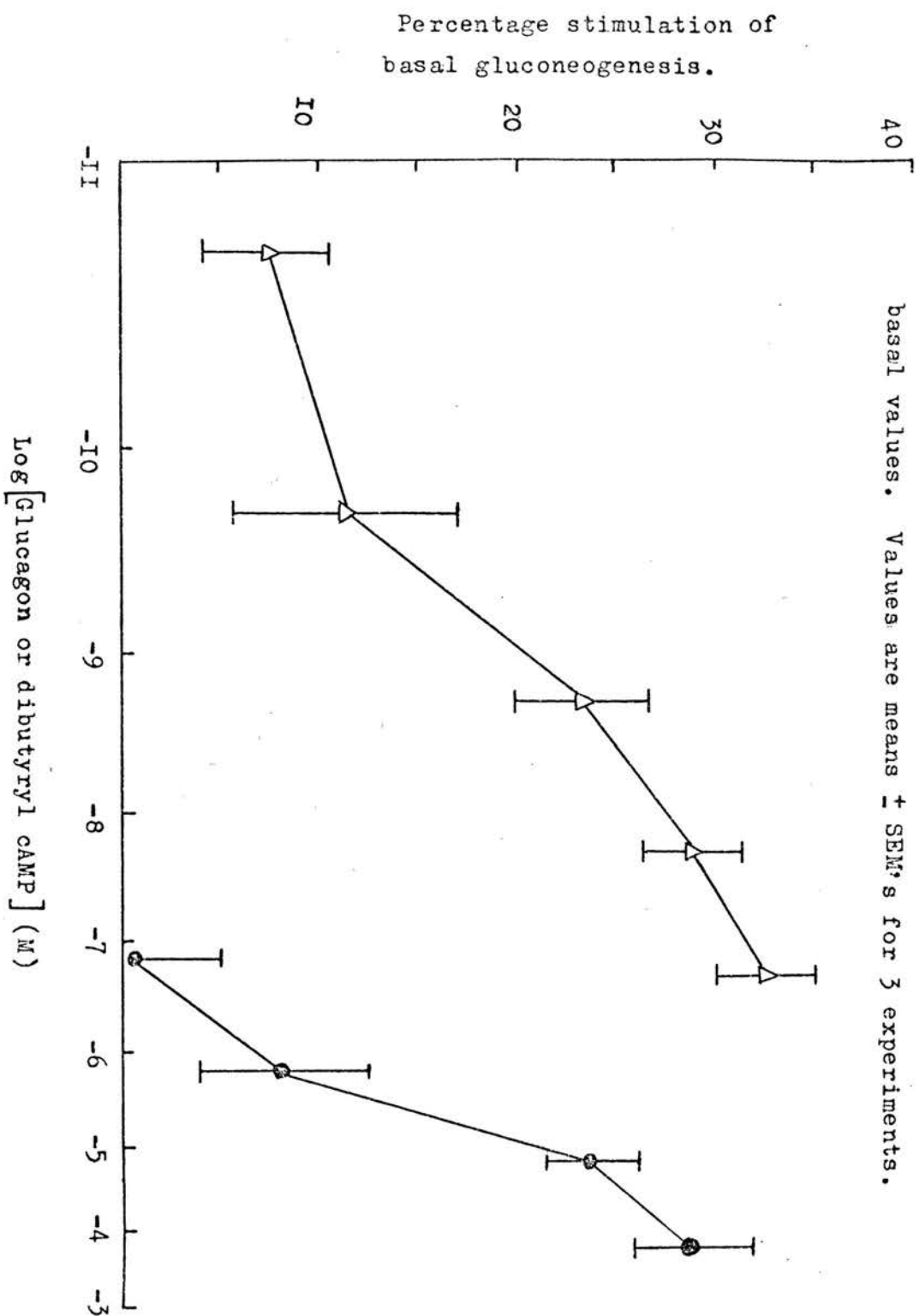


TABLE 6.3.      Stimulation of gluconeogenesis by glucagon and dibutyryl cyclic AMP.

Hepatocytes from 24h starved chickens were incubated for 30 minutes in the presence or absence of glucagon or dibutyryl cyclic AMP. Results are presented from 4 experiments. Each consisted of dihydroxyacetone, fructose and lactate with and without glucagon or dibutyryl cyclic AMP. Values are mean percentage stimulations of substrate only rates of gluconeogenesis  $\pm$  SEM's.

<u>Substrate</u>	<u>Concentration (mM)</u>	<u>PERCENTAGE STIMULATION</u>	
		<u>1.9nM Glucagon</u>	<u>13<math>\mu</math>M Dibutyryl cyclic AMP</u>
Dihydroxyacetone	0.5	14.7 $\pm$ 2.5	16.4 $\pm$ 3.4
Fructose	1.0	21.9 $\pm$ 4.8	23.1 $\pm$ 4.7
Lactate	2.5	13.9 $\pm$ 2.1	16.9 $\pm$ 3.6

contact with isolated rat hepatocytes is 3 minutes (Barnabei, et al., 1976), its degradation may prevent continued stimulation of glucose production at low concentrations of glucagon. Both processes are also stimulated by dibutyryl cyclic AMP to the same maximum extent. The study of the hormonal sensitivity of isolated chicken hepatocytes has shown that glucagon increases the intracellular cyclic AMP content, causing up to an 18-fold stimulation on hepatocytes from both fed and starved chickens (Anderson & Langslow, 1975; D.R. Langslow, personal communication). Although other requirements need to be fulfilled, the results suggest that glucagon stimulated both glycogenolysis and gluconeogenesis in chicken liver through cyclic AMP.

In the fed rat, glucagon stimulates glycogenolysis by simultaneously increasing glycogen phosphorylase activity and inhibiting glycogen synthetase, and the effects on these enzymes operate through a cyclic AMP-dependent protein kinase (Park & Exton, 1972). The observation that the glucagon effect is greatest on hepatocytes with high initial glycogen contents (Fig. 6.1) has also been made for isolated rat hepatocytes (Wagle, 1974; Hers, 1976). Chicken hepatocytes are similar to rat hepatocytes in other ways. The maximal stimulation of glycogenolysis is similar to the values from isolated rat hepatocytes (Johnson, et al., 1972; Garrison & Haynes, 1973; Wagle, 1975). Half-maximal glucagon effects on rat hepatocytes occur with  $10^{-10}$  -  $10^{-7}$  M (Garrison & Haynes, 1973; Wagle, 1975) and the value reported here ( $9.3 \times 10^{-9}$  M) compares favourably with the most sensitive isolated hepatocyte preparation.

Phosphodiesterase inhibitors, such as caffeine and theophylline, are often used to potentiate the actions of glucagon. Although glucagon-stimulated production of cyclic AMP was potentiated, caffeine



and theophylline decreased glucagon-stimulated glycogenolysis. Glycogenolysis by rat liver slices was inhibited by caffeine (10 mM) and this correlated with an inactivation of glycogen phosphorylase (Miller, et al, 1974). The effect observed in chicken hepatocytes may be more complex as caffeine also inhibits gluconeogenesis from lactate (D.R. Langslow, personal communication). This agrees with observations on rat hepatocytes that theophylline inhibits gluconeogenesis (Tolbert, et al, 1973). Recent evidence suggests that caffeine may nonspecifically affect glycogenolysis and gluconeogenesis by altering adenylate contents and oxygen consumption (D.R. Langslow, personal communication).

Hepatocytes isolated from starved rats exhibit half-maximal stimulation of gluconeogenesis with  $1.8 \times 10^{-7}$  M glucagon (Garrison & Haynes, 1973) and for the perfused rat liver is in the range of  $10^{-10}$  -  $10^{-9}$  M (Exton, et al, 1971; Lewis, et al, 1970). Thus the sensitivity of isolated hepatocytes from starved chickens appears greater than that of rat preparations. The maximal glucagon effects on the perfused liver from starved rats are double that in isolated rat hepatocytes (Williamson, et al, 1969C; Johnson, et al, 1972; Claus, et al, 1975; Siess & Wieland, 1975). The maximal glucagon stimulation of gluconeogenesis obtained in this study is slightly less than that of isolated rat hepatocytes. This does not reflect receptor damage during the preparation of chicken hepatocytes as the maximal effects of glucagon and dibutyryl cyclic AMP were similar (Fig. 6.5). Serum addition increased the extent of glucagon stimulation of gluconeogenesis by isolated rat hepatocytes (Siess & Wieland, 1975) and serum is often added to isolated liver perfusates. This may explain the discrepancy between isolated liver perfusion and isolated hepatocytes. The serum factor responsible has not yet been identified.

As the rate of basal gluconeogenesis from glycerol is low, probably due to the effects of the low ATP/ADP ratio on glycerokinase (see Chapter 5), the stimulation by glucagon may result in a more efficient conversion of glycerol to glucose in vivo. The stimulation of gluconeogenesis from fructose, glycerol and DHA (Table 6.3) suggests that glucagon can control the rate of at least one reaction above the level of triose phosphate dehydrogenase. Glucagon stimulates reaction(s) between triose phosphate dehydrogenase and glucose production in rat liver (Veneziale, 1972; Blair, et al, 1973; Garrison & Haynes, 1973; Tolbert & Fain, 1974). Evidence from metabolic crossover point studies and measurement of enzyme activities suggests that either fructose diphosphatase or glucose-6-phosphatase activity (or both) is increased. Conversely a change in flux towards glucose synthesis could be due to a decreased activity of glycolytic enzymes especially phosphofructokinase. This would lower the rate of substrate cycling and stimulate glucose synthesis. Although several authors have suggested that one specific reaction is affected, the concerted alteration of several enzyme activities would give similar effects. The addition of cyclic AMP to homogenates does not influence the rates of the enzymes mentioned above. As dibutyryl cyclic AMP stimulated gluconeogenesis in isolated chicken hepatocytes to the same extent as glucagon, increased gluconeogenesis is unlikely to be due to a direct effect of glucagon-produced cyclic AMP on specified glycolytic and gluconeogenic enzymes. The mechanism of stimulation may involve cyclic AMP interactions with other enzymes or metabolites, which then in turn directly stimulate gluconeogenesis.

In the rat liver glucagon also stimulates the net rate of conversion of pyruvate to PEP (Park & Exton, 1972; Blair, et al, 1973). This is either due to the stimulation of pyruvate carboxylase, phosphoenolpyruvate

carboxykinase or mitochondrial transport systems. The inhibition of pyruvate kinase (Feliu, et al, 1976) may play an extremely important role by decreasing wasteful cycling of PEP. In the rat the percentage stimulation of gluconeogenesis from lactate, pyruvate and alanine is greater than that from fructose and DHA (Garrison & Haynes, 1973; Tolbert & Fain, 1974) whereas in the chicken the stimulations are similar (Table 6.3). This difference may be explained either by the control point postulated between lactate and DHA (see Chapter 5), being insensitive to glucagon, or by the control point between triose phosphate dehydrogenase and glucose production being more rate limiting than the earlier reactions. Each would result in similar stimulation from lactate and DHA. No matter which of these possibilities holds, the mechanism of control of gluconeogenesis differs between rats and chickens. This may be due to the mitochondrial location of phosphoenolpyruvate carboxykinase either resulting in the reactions between pyruvate and PEP being insensitive to glucagon or resulting in the reactions not being rate limiting when compared to the site of control at hexose phosphate level.

The further investigation of glucagon effects on gluconeogenesis will require the study of crossover points and enzyme activities to locate the exact site(s) of glucagon action. Insulin antagonises glucagon effects on rat liver (Park & Exton, 1972) but is ineffective on glucagon action on isolated chicken hepatocytes (Anderson & Langslow, 1975). Whether this is due to a lack of insulin receptors in chicken liver, lack of insulin effect on glucose metabolism or to damage of the receptors during isolation remains to be determined. Glucagon effects in vivo may be complicated by interactions with circulating insulin but its actions observed in vivo (Langslow, et al, 1970) can largely be explained by effects at hepatic level.

SUMMARY

Isolated chicken hepatocytes have been shown to be sensitive to glucagon. Their sensitivity to this hormone is similar to that of the isolated rat hepatocyte although the sites of action on glucose production may not be identical.

By affecting either glycogenolysis or gluconeogenesis, glucagon stimulated glucose release from endogenous sources. Gluconeogenesis from added precursors was also stimulated by glucagon. The sensitivity of glycogenolysis and gluconeogenesis from added precursors to glucagon was similar. Effects on both processes are discernible with  $10^{-10}$  M glucagon and are maximal with  $10^{-6}$  M. As dibutyryl cyclic AMP mimicked the glucagon effects, and glucagon increased hepatocyte cyclic AMP content, it is probable that glucagon action is mediated through cyclic AMP production. The sites of action appear to be phosphorylase activity and between triose phosphate dehydrogenase and glucose production. The lack of apparent effect between pyruvate and PEP has been described, and the possibility that it reflects a true difference in control of gluconeogenesis discussed.

## CHAPTER 7

## CONCLUDING DISCUSSION

## CHAPTER 7 CONCLUDING DISCUSSION

Investigations in vitro require an uncomplicated system that yields reproducible results. Modification of existing procedures permitted the isolation of chicken hepatocytes by a simple and rapid technique. Many morphological and physiological characteristics of the isolated chicken hepatocyte were identical to the whole liver and the isolation procedure did not alter the ultrastructure of hepatocytes seen under the electron microscope. Several methods for the isolation of rat hepatocytes have resulted in a decreased  $K^+$  content compared to whole liver. Chicken hepatocytes prepared by this method also contain less  $K^+$  than whole liver. However, as this is to a large extent rapidly reversed during incubation, decreased  $K^+$  concentrations are unlikely to impair hepatocyte function.

The lack of a diaphragm in birds results in rapid anoxia before perfusion and subsequently in the low ATP/ADP.AMP ratio of the isolated hepatocyte. Anoxia also occurs during the isolation of pigeon liver but can be prevented by the use of an artificial oxygenation system (Söling, et al, 1973). The inclusion of an oxygenation system during the preparation of chicken hepatocytes might profitably be expected to maintain physiological ATP/ADP.AMP ratios. However, apart from the specific case of the low rate of gluconeogenesis from glycerol, there is no evidence that low ATP/ADP.AMP ratios impair hepatocyte metabolism. As discussed later, anoxia may act as a stimulus for rapid glycogenolysis.

By comparing alterations in the metabolic and morphological characteristics of hepatocytes during incubations, hepatocytes have

been shown to undergo degenerative changes at 40°C. The permeability to trypan blue was a reliable indicator of loss of functional abilities of the hepatocyte. This was not primarily due to lack of substrate as exogenously supplied substrates did not prevent degeneration. The breakage of cell-cell contacts may result in greater parenchymal cell instability than is observed in vivo. The changes may also represent latent effects of the mechanical treatment during isolation or of collagenase (or protease contaminants) bound to membranes. This latter possibility could be investigated by the addition of collagenase or protease antibodies although the initial contact with collagenase might be sufficient to cause all subsequent effects.

Hepatocytes from starved chickens were damaged during incubation at a greater rate than those from fed chickens. During starvation of rats, the hepatocyte protein (Cornell & Filkins, 1974) and lipid content (Wimhurst & Manchester, 1973) decreases and these depletions could result in the increased fragility of hepatocytes from starved chickens. The changing proportion of intact and inactive hepatocytes complicates the interpretation of results. Incubations of only 30 minutes proved suitable for studying most of the aspects of gluconeogenesis reported here and resulted in only small increases in the proportion of stained cells.

Collagenase activity greatly affected the quality and quantity of preparations. This has also been reported for rat hepatocytes (Berry, 1974). Batches of collagenase differ in their content of  $\text{Ca}^{++}$  and proteases and both can alter the collagenase activity. Consistency was maintained throughout the last ten months of my project

by the use of a single batch of collagenase. Fixed digestion times with collagenase should be avoided, and as with isolated fat cells, art must triumph over science so that the appearance of the digestion mixture should be used to indicate when digestion is complete.

Rapid glycogenolysis resulted in greater endogenous glucose release from hepatocytes from fed than from starved chickens. Stimulated phosphorylase activity, and hence glycogenolysis, was probably due to the anoxia during hepatocyte preparation. The incubation of hepatocytes in high  $K^+$ -KRB decreased glycogenolysis, suggesting that the lowered hepatocyte  $K^+$  content may also be a factor responsible for stimulated phosphorylase activity.

The addition of gluconeogenic precursors stimulated glucose production by both fed and starved chicken hepatocytes. Whether the increased glucose production represents net gluconeogenesis with fed chicken hepatocytes would only be determined by the use of radio-actively labelled precursors. Qualitatively the effects of precursors on gluconeogenesis by starved chicken hepatocytes are largely similar to the increased plasma glucose concentration of chickens caused by injection of these precursors in vivo (Davison & Langslow, 1975). Only glycerol and alanine are less effective in vitro than in vivo and the lowering of the hepatocyte ATP/ADP.AMP ratio with its inhibitory effect on glycerokinase can explain the low rate of glycerol gluconeogenesis in vitro. In vivo alanine may be more effective due to the interactions with other substrates (such as glycerol) and glucagon. The effectiveness of alanine in vivo was most noticeable after three hours treatment (Davison & Langslow, 1975) and the presence of alanine in plasma may cause the induction of alanine aminotransferase



activity. Reduced substrates (e.g. sorbitol, xylitol, and glycerol) potentiated glucose production when added with an oxidised substrate (e.g. pyruvate). These synergistic effects may be relevant to the degree of glucose production in vivo. Gluconeogenesis from xylitol, although occurring at a low rate, occurs via xylulose-5-phosphate and suggests that, unlike previous reports (Heald, 1963), the enzymes of the pentose phosphate pathway may indeed be present in chicken liver.

The ordering of effectiveness of gluconeogenic precursors in chicken and rat hepatocytes are similar except for glycerol and the low rate of gluconeogenesis from alanine and pyruvate compared to lactate. These differences can be explained by the low activity of alanine aminotransferase and the mitochondrial location of phosphoenolpyruvate carboxykinase. All glucose synthesised was released from the cells and the insignificant rate of glycogen synthesis was probably a result of the postulated initial activation of phosphorylase (Hue, et al, 1975). Although the use of radioactively labelled glucose might show glucose incorporation into glycogen, the turnover rate may be too high to allow net synthesis. It may prove possible to increase glycogen deposition by improvement of the conditions (oxygenation) during hepatocyte isolation. Artificial oxygenation during cell preparation may maintain high ATP/ADP.AMP ratios and prevent phosphorylase activation.

Glucagon stimulated glycogenolysis and gluconeogenesis at physiological concentrations. Although the same pattern was always observed, the magnitude of stimulation depended upon the quality of individual preparations. Trypan blue staining and other means of viability assessment give no indication of the relatively small

alterations of hepatocyte membrane integrity that constitute hormone receptor damage. Some collagenase batches destroyed glucagon receptors either due to inefficient digestion of the matrix and subsequent forceful mechanical separation or to protease contamination. Each batch was analysed for receptor damage.

On the basis of the stimulation of gluconeogenesis from different substrates, glucagon is postulated to stimulate a reaction between triose phosphates and glucose release. The site of action between pyruvate and PEP, present in rat liver, was not observed in the present study. This may suggest a different site of glucagon action in chicken liver compared to rat liver or may merely be due to limitations in the means of investigation used here.

In summary, the properties of isolated chicken hepatocytes compare favourably with the whole liver. This similarity has permitted a study of the mechanisms of gluconeogenesis and its control by glucagon to be undertaken. The results indicate that the mitochondrial location of phosphoenolpyruvate carboxykinase gives rise to many of the unique features of chicken liver compared to rat liver. Although the regulation of gluconeogenesis at mitochondrial level may be altered, it is mainly the limitations of hydrogen transfer reactions during gluconeogenesis that alters the metabolic profile of chicken liver. Not only is the isolated chicken hepatocyte suitable for studying the mechanism of gluconeogenesis in the domestic fowl but should enable the interactions and integration of carbohydrate, lipid and protein metabolism to be studied.

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## APPENDIX I

### STATISTICAL METHODS

Results were expressed in the form:-

mean value  $\pm$  standard error of the mean.

The standard error of the mean (SEM) was calculated from the general formula

$$SEM = \sqrt{\frac{\sum (m-x)^2}{n(n-1)}}$$

where "x" represents the observed value,

"m" is the arithmetic mean of the observed values,

"n" is the number of observations,

"n-1" is the number of degrees of freedom.

The significance of the difference between means was determined by the Student's "t"-test. The value of "t" was calculated using the formula

$$t = \frac{m_1 - m_2}{S \cdot \sqrt{\frac{1}{n_1} + \frac{1}{n_2}}}$$

where "m<sub>1</sub>" and "m<sub>2</sub>" are the means of the two sets of observations, and "n<sub>1</sub>" and "n<sub>2</sub>" are the number of observations in each case. Values of "s" are obtained by taking the square root of the mean square deviation of the two groups (s<sup>2</sup>)

$$s^2 = \frac{\sum (m_1 - x_1)^2 + \sum (m_2 - x_2)^2}{n_1 + n_2 - 2}$$

The probability, P, that the difference between any two means was

significant, was obtained from the relevant value of "t" and the number of degrees of freedom ( $n_1+n_2-2$ ) by reference to probability tables. The difference between two means was considered significant when  $P \leq 0.05$

## **APPENDIX 2**

### **SOURCES OF ANIMALS, CHEMICALS AND ENZYMES**

ANIMALS

Chickens (Thornber 909's, males) were obtained from the Poultry Research Centre, Kings Building, West Mains Road, Edinburgh between the ages of 3 and 6 weeks. They were then housed under strictly controlled conditions of food (commercial chicken starter pellets), light and temperature in the Wellcome Animal House, Royal (Dick) School of Veterinary Studies, Summerhall, Edinburgh. To permit acclimatisation chickens were not used for studies during the first two days following rehousing.

CHEMICALS AND ENZYMESArmour Pharmaceutical Co., Eastbourne, Sussex

bovine serum albumin, fraction V

Boehringer Corporation, London

ADP (disodium salt)

AMP (disodium salt)

ATP

adenosine

L-(+)-aspartic acid

dibutyl cAMP

$\alpha$  ketoglutaric acid (disodium salt)

NADH (grade II)

phosphoenolpyruvate (monopotassium salt)

sodium pyruvate

collagenase (grade II) (E.C. 3.4.24.3)

glucose oxidase (E.C. 1.1.3.4.)

glycerokinase (E.C. 2.7.1.30)

hyaluronidase (E.C. 3.2.1.35)

lactate dehydrogenase (E.C. 1.1.1.27)



malate dehydrogenase (E.C. 1.1.1.37)

myokinase (E.C. 2.7.4.3.)

peroxidase (E.C. 1.11.1.7)

pyruvate kinase (E.C. 2.7.1.40)

British Drug Houses, Ltd., Poole, Dorset

4-amino phenazone

citric acid

fumaric acid

glycerol

glycine

L-malic acid

L-serine

sorbitol

succinic acid

Xylitol

Gurr, High Wycombe, Bucks.

Trypan blue

Hopkins & Williams, Romford, Essex

L-alanine

D-fructose

repelcote

Koch-Light Ltd., Coinbrook, Bucks

2,5-diphenyloxazole (PPO)

1,4-di(5-phenyloxazolyl)benzene (POPOP)

Sigma (London) Ltd., Kingston-upon-Thames, Surrey

dihydroxyacetone

F.L.E.-50

D-glyceraldehyde

hydroxypyruvic acid (monolithium salt)

L-(+)-lactic acid

phosphocreatine (disodium salt)

apyrase (grade II) (E.C. 3.6.1.5.)

creatine phosphokinase (type I) (E.C. 2.7.3.2.)

The Radiochemical Centre, Amersham, Bucks

1-<sup>14</sup>C glycerol

Glucagon (porcine) was a kind gift of Eli Lilly & Co. Ltd., Indianapolis, U.S.A. All other chemicals apart from those specified above were obtained as Analar grade from British Drug Houses, Ltd.

All solutions were made up with distilled water, and in the case of flame emission spectrophotometry with deionised, distilled water. Glassware was cleansed by overnight soaking in detergent solution (Decon), thoroughly rinsed in tap water, followed by three rinses in distilled water and oven dried.